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STUDIES OF THE SKIN REACTIONS TO THE SPECIFIC SOLUBLE SUBSTANCES OF THE PNEUMOCOCCUS TYPES I AND II.

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IN 1929, Tillett and Francis showed that in patients suffering from pneumonia there developed at the time of crisis, or soon after it, skin-sensitiveness to homologous pneumococcal specific soluble substance, and they believed that a patient's capacity to react was intimately associated with recovery from infection, and with the presence of type-specific antibodies in the circulating blood. Alston, Galbraith and Stewart (1930) confirmed the occurrence of cutaneous sensitiveness to S.S.S.* type I in patients convalescent from type I pneumonia, but they noted that several of their cases gave a reaction at a period of 3 to 5 weeks after the crisis when no agglutinating action of the serum could be detected.

Specimens of type I and type II S.S.S. were prepared by one of us (A. L.) in 1930–31, and have been used for the tests and experiments to be reported. Reports on cutaneous sensitiveness to these substances have been made by Francis and Tillett (1930, 1931), Finland and Sutliff (1931, *a*, *b*, 1932), and Sutliff and Finland (1932). Our results confirm the findings of these investigators in some respects but differ from theirs in others.

The type I substance was prepared by the method of Heidelberger, Sia and Kendall (1930). By the ninhydrin and the xanthoproteic reactions no protein was detectable in this specimen. The material did not completely dissolve in 0·85 per cent. NaCl solution, and as recorded by Heidelberger, Sia and Kendall, required alkali (0·6 per cent. N/1 NaOH) for complete solution. The type II specific soluble substance was obtained by the method of Heidelberger and Avery (1923). The ninhydrin reaction was negative, and the specimen was completely soluble in 0·85 per cent. saline solution.

The activity of the specimens as type-specific haptenes was determined by ring precipitation tests with saline solutions of the carbohydrates and type-specific pneumococcal antisera. In Table I it is shown that at the time

* S.S.S. denotes specific soluble substance.

of preparation the specimens were very highly and specifically reactive. The effect of *storage* and *heat* on the specimens was tested. For the former, precipitation tests were carried out after the carbohydrates had been kept at room temperature in the dry state for almost 18 months. The results of these tests also are recorded in Table I, and show that some loss of activity occurred in both specimens. The effect of exposure for a short period to heat was observed on the specimens 18 months after preparation. Solutions of the carbohydrates were heated at 99° C. in a water-bath for 15 minutes and at 120° C. in an autoclave for the same period. This treatment did not reduce the activity of the carbohydrates in forming a precipitate with specific antiserum, except in the case of the type II substance after exposure to 120° C.

TABLE I.

S.S.S.			Dilutions of S.S.S.											Type of serum.
Type.	Age (mths.).	Heat.	1 in 500.	1 in 5000.	1 in 50,000.	1 in 500,000.	1 in 1,000,000.	1 in 2,000,000.	1 in 3,000,000.	1 in 4,000,000.	1 in 5,000,000.	1 in 7,000,000.	1 in 15,000,000.	1 in 20,000,000.	
I	Fresh	Unheated	++++	+++	++	++	..	..	..	..	++	+	±	±	I
I	18	,,	++++	..	..	..	..	..	..	..	+	+	−	−	I
I	,,	99° C.	..	..	..	..	..	..	..	..	..	+	..	..	I
I	,,	120° C.	..	..	..	..	..	..	..	..	..	+	..	..	I
II	Fresh	Unheated	++++	+++	++	++	+	+	..	±	..	..	..	..	II
II	18	,,	..	..	..	+	+	±	−	−	..	..	..	..	II
II	,,	99° C.	..	..	..	..	+	±	..	..	..	..	..	..	II
II	,,	120° C.	..	..	..	+	±	−	..	..	..	..	..	..	II

Neither of the two specimens gave a reaction with a heterologous type-specific pneumococcal antiserum.

++++ = thick ring formed immediately, settling in a few minutes to the foot of the tube; +++ = thick ring, settling less rapidly; ++ = definite ring, later becoming diffused; + = faint but definite ring; ± = distinct cloudiness in supernatant layer, without ring-formation.

REACTIONS OF THE SKIN TO INTRACUTANEOUS INJECTION OF THE SPECIFIC SOLUBLE SUBSTANCES.

Description of the Reactions.

The types of reaction produced by the injection of the carbohydrate preparations into the skin were the same in the case of both substances, and in each case they could be described as a primary and a secondary type.

The primary reactions consisted of a wheal and erythema, and commenced to appear from 5–15 minutes after the intradermal injection was made, developed to a maximum during the course of 10–30 minutes and subsequently receded. The largest area of erythema produced was 4 in. in diameter. In the case of the more severe reactions subcutaneous œdema with tenderness, developing during the time of the superficial reaction and affecting a wider area, remained for up to 24 hours. In a few instances the skin over the whole

of this area of subcutaneous œdema became erythematous, and remained so for several hours.

The secondary reactions were distinct from the primary in nearly all cases. They appeared from 2–5 hours after the injection, developed to a maximum in about 2–12 hours longer, and had disappeared by 8–48 hours after the time of injection. They consisted of erythema of a deeper red colour than in the earlier reactions and without a central wheal or pallor, and were accompanied in the greater degrees by subcutaneous œdema and tenderness. The largest secondary lesion extended about 6 in. by 3. Almost always when an individual showed both types of reaction the erythema of the first had disappeared by the time that the second developed.

TABLE II.—*Skin Tests with S.S.S. II in Persons Without Pneumococcal Infection: Primary Reactions.*

Age.	Positive reactors.										Negative reactors.		Total tested.
	++++.		+++.		++.		+.		All positives.				
	Numbers.	Percentage.*	Numbers.	Percentage.	Numbers.	Percentage.	Numbers.	Percentage.	Numbers.	Percentage.	Numbers.	Percentage.	Numbers.
0 – 1½ years	0	0	0	0	0	0	0	0	0	0	15	100	15
1½–12 ,,	0	0	5	5·3	22	23·4	20	21·3	47	50	47	50	94
Above 12 years	20	11·6	36	20·9	39	22·6	36	20·9	131	76	41	24	172

* " Percentage " means the percentage of all tested in the same age-group.

Control injections of physiological saline caused in some persons a reaction of minor degree similar to the erythema of the primary reaction with the carbohydrate. In these cases the recorded reading of the test was altered to take account of the reaction to saline alone. With the type I substance 0·6 per cent. N/1 NaOH required to be added to the saline, and this mixture produced in many persons reactions as large as any due to the solution of carbohydrate.

Reactions Obtained.

With type II S.S.S. the skin test was made with 0·05–0·1 mgm. dissolved in 0·1 c.c. saline, and a primary reaction occurred in all of four persons convalescent from type II lobar pneumonia.

Reactions were examined with the same type of carbohydrate in three groups of persons not suffering or convalescent from pneumococcal infections; (1) infants (0–18 months) in hospital, (2) children (1½–12 years) in hospital, and (3) adolescents and adults (12–40 years), of whom most were in good health. In all groups 281 persons were tested, and 178 (63 per cent.) gave primary reactions exactly similar to those previously produced in pneumonia convalescents with the type I or type II carbohydrate. In Table II it is

shown that no primary reactions occurred in infants, none was of maximum intensity in children under 12 years, and that in the group of adolescents and adults the largest percentage of reactors and all the most intense reactions occurred.

The preparation of type I S.S.S. was injected intradermally in 3 persons convalescent from lobar pneumonia of other types than I, and in 7 who showed no pneumococcal infection. The amount injected was 0·05 mgm. dissolved in 0·1 c.c. physiological saline containing 0·6 per cent. N/1 NaOH. In these persons the alkaline saline alone did not produce a reaction, but the carbohydrate caused a primary reaction in 1 convalescent and in 5 healthy subjects, and a secondary reaction in all of the nine in whom it was looked for.

As regards *animals*, in the case of healthy rabbits, of 20 injected intradermally with 0·1 mgm. S.S.S. I and of 5 injected with the same dose of S.S.S. II, none showed a reaction. Several guinea-pigs and mice were tested with the type II substance and none reacted.

In several normal persons simultaneous tests were carried out with different doses of S.S.S. II. The results showed that the degree of primary reactions is roughly proportional to the amount injected (Table III), and this increases the significance of the different degrees of reaction obtained in different persons with a constant dose.

Fifty persons were tested as regards their primary reactions to 0·05 mgm. S.S.S. II on two occasions, with an interval of 5 to 12 (mostly 5 to 7) months between the tests. These persons are in the group "above 12 years" in Table II, in which only the earlier reaction obtained is included. The comparison of the two results is made in Table IV and is between negative and positive results, without attention to degrees of reaction. The two reactions were the same in 80 per cent. of cases, and an analysis (Table IV) of those in whom the second result differed from the first shows again that the number of reactors increases with age, but it also shows that a few persons were found first to be positive and afterwards negative.

TABLE III.—*Tests with Different Dilutions on Same Person. Type II S.S.S.*

Subject.	Amount of S.S.S.						
	1 mgm.	0·1 mgm.	0·05 mgm.	0·04 mgm.	0·01 mgm.	0·002 mgm.	0·001 mgm.
	Dilutions of S.S.S.						
	1 in 100.	1 in 1000.	1 in 2000.	1 in 2500.	1 in 10,000.	1 in 50,000.	1 in 100,000.
J. H—	..	++++	..	++++	..	+++	..
J. K—	..	++	++	..	++	+	−
A. H—	..	++++	+++	..	++	++	++
H. S—	..	+++	..	+++	..	..	..
M. C—	..	++++	..	++++	..	..	..
H. W—	..	++++	..	++++	..	..	..
M. A—	+	..	−	..	..	..	..
A. L—	..	++	..	+	..	..	..
E. S—	−	−	−	..	..	..	..

All readings taken ½ hour after test. Controls negative.

The number of + signs indicates the degree of the reaction from the largest observed (4 in. or more in one diameter) to the smallest which lasted for at least ten minutes, in the case of the primary reactions; or the smallest which were observed at any time during the 5–24 hours' period in the secondary ones.

TABLE IV.—*Comparison of Two Tests in the Same Person. 0·05 mgm. Type II S.S.S.*

Number of persons tested		50
2nd result same as 1st	both positive 35 both negative 5	40 = 80 per cent. of persons tested.
2nd result different from 1st	1st positive, 2nd negative, 3 1st negative, 2nd positive 7	10 = 20 per cent. of persons tested.

Of 12 negative at 1st test, 7 (= 58 per cent.) were positive at 2nd test.
Of 38 positive ,, 3 (= 8 per cent.) ,, negative ,,

When injections were repeated *several* times, especially at *short intervals*, it was found that a reaction was followed by one of smaller degree or by an absence of reaction, and that a negative result was not altered by several injections of S.S.S. at short intervals. Such observations were made in human beings and animals with type I or type II S.S.S. or both. Although a loss of reactivity of the S.S.S. as regards precipitation reaction has been recorded (Table I), the degree and rate of such loss cannot be taken to account for the much greater and more rapid loss of reactivity to the substance which these subjects showed. All the persons on whom repeated tests were made are included in Table V, (*a*) and (*b*). It may be stressed that with type II S.S.S. two subjects (1 and 2) were injected 12 and 6 times respectively between June 14 and July 28 without producing a reaction. In one subject (No. 6 in Table V, (*b*)) no reaction was found in May, 1931, a + reaction in November and no reaction next May. The intervals between tests were long, and this case does not, therefore, make an exception to the general finding of a decrease in reactivity when tests were repeated at short intervals. As regards *animals*, two rabbits were injected intradermally twice a week during 8 weeks with S.S.S. type I, the total amount injected being 1·7 mgm., and no primary reaction was observed, although a faint secondary reaction occasionally appeared at 3–6 hours and lasted for up to 24 hours. In addition 8 rabbits vaccinated with type I or type II pneumococci, as described later, were tested by intradermal injection of 0·15 or 0·075 mgm. S.S.S. homologous to the vaccine on each of ten occasions in three weeks and no primary reaction occurred, although there were occasional faint secondary lesions.

Active and passive pneumococcal immunization was produced in men and animals—non-reactors—to know whether a positive reaction could be thus produced. Three rabbits were vaccinated with type I and three with type II pneumococcus. The serum agglutination titre rose to 1 in 64 in 3 cases, 1 in 32 in 2 and 1 in 8 in 1, but no primary skin reaction was observed during ten weeks' testing, although in all but one a delayed, secondary type of reaction occurred during the period 2–48 hours after injection. The frequency and size of these reactions increased during the course of the vaccination, although the animal which showed no reaction of any kind was one of those with the most strongly agglutinating serum.

Vaccination of a human subject with type II pneumococci similarly produced no alteration of skin reaction, which remained a very weak one throughout.

Rabbits were given intravenous, subcutaneous and intracutaneous injections of Lederle's concentrated antipneumococcal serum (types I and II), and while

TABLE V.—*Tests Repeated Several Times on the Same Person.*

(*a*) Type I S.S.S. : Dose as recorded.

Dose.		1931.						1932.
		March.	April.					September.
		16.	8.	10.	14.	15.	16.	..
(1) J. M. A—	0·3 mgm.	+++	..	..	..	+++	++	..
	0·2 ,,	..	..	..	..	..	++	..
	0·1 ,,	..	++++	++	..	..	++	−
	0·05 ,,	..	..	+++	+ (2)	..	++	..
(2) A. L—	0·1 ,,	..	+++	..	..	..	..	..
	0·05 ,,	..	..	++	−	..	..	..
(3) E. S—	0·1 ,,	..	+++	..	..	..	..	−
	0·05 ,,	..	..	++	−	..	..	..

(*b*) Type II S.S.S. : Dose 0·05 mgm.

	1931.			1932.																
	May.	September.	November.	May.	June.					July.						September.					Oct.
	..	..	..	..	14.	16.	17.	18.	20.	11.	25.	26.	27.	28.	29.	..	26.	28.	29.	30.	3.
(1) J. M. A—	++,+	+ (2)	+ (2)	−	−	−	−	−	−	−	−	− (2)	− (3)	−	..	− (2)	..	..	..	..	..
(2) E. S—	−	−	− (2)	−	−	−	−	−	−	−	−	..	..	..	..	− (2)	..	..	..	..	..
(3) A. H—	++++	..	+++	..	+++	..	..	..	..	..	..	..	..	..	++ (2)	++ (2)	..	..	..	..	..
(4) J. K—	..	++	++	++	..	..	..	..	..	..	..	..	..	..	+ (2)	+	..	..	..	..	..
(5) J. W—	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	+++	++	+	+	+
(6) C. P. S—	−	..	++	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
(7) J. H—	++++	++++	++++	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

the serum agglutination titre rose to 1 in 64, no skin sensitivity to S.S.S. I or II was found at the site of subcutaneous or intracutaneous injection of serum or elsewhere.

In a human subject, a non-reactor to S.S.S. II, 5 c.c. of Lederle's serum was injected in portions intracutaneously and subcutaneously during 24 hours, but neither at the site of intracutaneous injection or elsewhere did sensitivity to S.S.S. II develop.

The converse experiment was carried out of injecting 0·5 c.c. of Lederle's serum intracutaneously in two human subjects who were sensitive to S.S.S. II, but as soon as the erythema produced by the serum injection had faded the skin was still sensitive to the carbohydrate.

Agglutination reactions and skin tests.—Among 40 healthy adults only 1 showed serum agglutinins to Pneumococcus II in higher dilution than 1 in 2. This individual gave a reaction (++) to S.S.S. II. Of the other 39, 27 were also reactors.

A comparison of the presence in the serum of *mouse-protective antibodies* against pneumococci with skin reactions pointed to an absence of positive correlation between the two. The method of testing mouse-protective power was that used by Alston and Stewart (1931) in the case of Subjects 5 and 6 in their paper. The results were that while the serum of four human positive reactors had high, and one non-reactor very low, mouse-protective power, a rabbit and a human subject in whom high protective power had been induced by active and passive immunization respectively, were non-reactors.

SECONDARY REACTIONS IN PERSONS NOT SHOWING PNEUMOCOCCAL INFECTION.

In many of the persons of all age-groups the test was observed during the period 5–24 hours after injection, as well as ½–1 hour. The secondary reactions and their relation to primary reactions are set out in Table VI. The secondary reaction was more frequent in older persons, and was much more frequent in those who showed a primary one than in those who did not.

TABLE VI.—*Secondary Reactions in Various Age-Groups.*

(*a*) Age-group 0–1½ years.
Tests read at ½–1 hour, 5 hours and 24 hours.
15 tests on 15 persons.
Negative at all readings, 15 = 100 per cent.

(*b*) Age-group 1½–12 years.
Tests read at ½–1 hour and at 24 hours.
90 tests on 90 persons.
Negative at all readings, 45 = 98 per cent. of 1st reading negatives.
,, ½–1 hour, positive at 24 hours, 1 = 2 per cent. of 1st reading negatives.
Positive at all readings, 3 = 7 per cent. of 1st reading positives.
,, ½–1 hour, negative at 24 hours, 41 = 93 per cent. of 1st reading positives.
Primary reactions = 48 per cent. of tests.
Secondary reactions (at 24 hours) = 4·4 ,, ,,

(*c*) Age-group above 12 years.

Tests read at ½–1 hour, 5–7 hours and 24 hours.

124 tests, some being repeat tests on the same person.

Negative at all readings, 34 = 83 per cent. of 1st reading negatives.

,, ½–1 hour, positive at 5–24 hours, 7 = 17 per cent. of 1st reading negatives.

Positive at ½–1 hour and positive at 5–24 hours, 39 = 47 per cent. of 1st reading positives.

,, ½–1 hour and negative at 5–24 hours, 44 = 53 per cent. of 1st reading positives.

Primary reactions . . . . . = 67 per cent. of tests.

Secondary reactions at 5 or 24 hours or both = 37 ,, ,,

,, ,, 24 hours . . . = 22 ,, ,,

Of all secondary reactions, 39 (= 85 per cent.) occurred with a primary reaction and 7 (= 15 per cent.) without a primary reaction.

(*d*) Percentage secondary reactions in age-groups :

0–1¼ years.	1¼–12 years.	Above 12 years.	
At 5 and 24 hours.	At 24 hours.	At 5 or 24 hours or both.	At 24 hours.
0	4·4	37	22

DISCUSSION.

Francis and Tillet (1930) confirmed, in general, by Finland and Sutliff (1931, *a*), found that although skin-reactions at the time of recovery from lobar pneumonia were limited to the homologous S.S.S., after repeated tests with heterologous pneumococcal carbohydrates reactions were obtained in certain instances to these heterologous substances as well, and they considered that the development of the heterologous reactions might be due to an *in vivo* combination of the S.S.S. injected intradermally with protein in the body. Finland and Sutliff (1931, *b*) stated also that this heterologous sensitiveness was not permanent, since some subjects lost it after a few weeks or months. We suggest that the heterologous reactions may be due to a re-appearance of reactions which were suppressed during the course of the disease, since we found 67 per cent. of reactors to S.S.S. II among persons over 1½ years, and since Finland and Sutliff (1931, *b*) consider the possibility of such suppression of reactions and quote other authorities for its occurrence. The temporary nature of the heterologous reactions which they found we consider may be a loss of reactivity due to repeated testing, as we recorded.

In the human subject and in rabbits we failed to produce primary skin reactions by active and passive immunization with the pneumococcus, but like Francis and Tillet (1931), we found in rabbits in such circumstances a late, secondary type of reaction.

SUMMARY AND CONCLUSIONS.

1. In 281 persons who had not recently suffered from a pneumococcal infection, sensitiveness (increasing with age) to intracutaneous injection of pneumococcal S.S.S. type II was found in 63 per cent.

2. Reactors re-tested after 5–12 months were still sensitive in 92 per cent. of cases.

3. Repeated tests at short intervals always produced a decrease, and in some cases a complete loss of sensitiveness.

4. In animals and human beings actively or passively immunized to pneumococci no immediate reactions could be produced by S.S.S. of homologous type. In rabbits, however, vaccination caused the development of a delayed form of reaction to the carbohydrate.

5. Skin sensitiveness (for immediate reactions) to the pneumococcal type-specific carbohydrates and circulating serum antibodies can each occur independently of the other, and our work does not support the suggestion that the skin reactions are associated solely with recovery from pneumonia.

6. In the normal persons tested with intracutaneous injections of S.S.S. type II a considerable proportion showed as well as, or independent of, the immediate reaction, a delayed reaction. This was also found to be much more frequent in the older persons.

While taking part in the work, one of us (J. M. A.) was in receipt of a grant from the Medical Research Council.

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THE HETERO-TRANSFER OF TWO FILTERABLE TUMOURS: AN INVESTIGATION BY MEANS OF RESISTANCE INDUCED BY EMBRYO TISSUE.

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THE Fujinami fowl-myxosarcoma can be propagated in ducks, both old and young (Fujinami and Hatano, 1929; Gye, 1931, 1932; Purdy, 1932, *a*), and the Rous fowl-sarcoma No. 1 can be propagated in ducklings (Purdy, 1932, *b*). In fowls both tumours are filterable, but only the Fujinami tumour can be transferred to ducks by means of filtrates. The Rous tumour can be transferred to ducks only when minced tissue is used, and then only to newly-hatched birds. Why this difference between the tumours? Can it be that

towards the agent of the Rous sarcoma adult and part-grown ducks are much more resistant than ducklings; or is there some other explanation? Obviously a fuller understanding of the hetero-transfer of tumours is important, and the importance is greatest when, as here, the tumours are filterable. For this reason it is proposed to describe and discuss some experiments in which the formation of tumours in ducklings previously inoculated with minced embryo has been compared with the formation of tumours in normal ducklings.

Years ago it was discovered that some transplantable tumours will not grow in mammals which have received an inoculation of embryonic or other normal tissue. A power of resistance is induced by the embryo, though not unless both embryo and tumour come from one and the same species of mammal. The phenomenon has attracted much attention, and has been studied with care by many workers. Most recorded observations have been made upon mice. Russell (1908) traced the non-development of tumours in resistant animals to the fact that the tumour-cells of the graft fail to become established, and he concluded that the tumour-cells fail simply because they are foreign to the host, and not at all because they have malignant properties. Rous (1910) confirmed Russell's conclusion when he showed that this artificially induced resistance is equally effective against grafts of normal embryonic tissue. To-day Russell's conclusion is generally accepted. More complete reference to the subject is unnecessary here, since Woglom (1913, 1929) has reviewed the literature in detail.

All the experiments to which attention will be drawn have been carried out on one general plan, and if certain technical details are mentioned now, much tiresome repetition will be avoided. All ducks and ducklings have been of the Khaki Campbell variety. Ducklings have never been more than 24 hours old at the beginning of the experiment in which they have been used. Mince has always been freshly prepared just before it has been needed for inoculation. Embryo mince has been made by clipping with scissors one or more complete embryos; the embryos have ranged in age from 9 to 14 days. Tumour mince has always been pumped free of the air-bubbles which become included during the mincing and make more difficult the accurate measurement of doses. In embryo mince air-bubbles rarely appear in sufficient numbers to cause trouble.

The first question is whether a duckling which has received an injection of fowl-embryo becomes resistant to a cell-free extract of fowl-grown Fujinami myxosarcoma. The answer was hardly in doubt. A form of resistance which is directed solely against the establishment of injected foreign cells could not be expected to prove effective against a filtrate which contains no cells. If the injection of embryo tissue rendered a duckling resistant to a filtrate, then the views at present held concerning the action of embryo would need modification. The question was put in experimental form in the following way:

On June 8th, 1932, 12 newly-hatched ducklings were divided into three groups. Each duckling of Group A was given 0·5 c.c. of minced fowl-embryo, and each duckling of Group B was given 0·5 c.c. of minced duck-embryo. In each duckling the mince was delivered deep into the muscles of the right leg. The ducklings of Group C received no embryo. On the 16th, 8 days after the embryo injections, all the ducklings of the three groups were given into the

muscles of the left leg 1·0 c.c. of a thrice-spun saline extract of a Fujinami tumour, which had been grown in a fowl. The resulting tumours are shown in Fig. 1, which summarizes the experiment. Tumours grew with equal readiness in the ducklings of all three groups. This result is regarded as significant, for, as will be seen from the results of experiments to be described later, the lack of resistance in the ducklings which had received embryo tissue cannot be attributed to unsuitability of the interval—8 days—allowed between the embryo and filtrate injections; nor can the dose of embryo be regarded as inadequate. The result has been confirmed in other experiments in which the tumour extracts have been actually filtered before use.

This failure of embryo tissue—both fowl and duck—to prevent the agent of the Fujinami tumour from infecting host-cells was to be expected for the reasons already given, but proof was necessary before it could be asked with

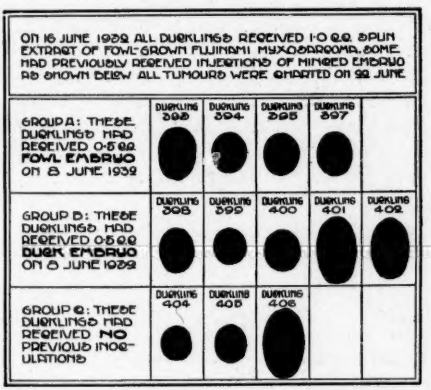
ON 16 JUNE 1932 ALL DUCKLINGS RECEIVED 1·0 C.C. SPUN EXTRACT OF FOWL-GROWN FUJINAMI MYXOSARCOMA. SOME HAD PREVIOUSLY RECEIVED INJECTIONS OF MINCED EMBRYO AS SHOWN BELOW. ALL TUMOURS WERE STARTED ON 22 JUNE.
GROUP A: THESE DUCKLINGS HAD RECEIVED 0·5 C.C. FOWL EMBRYO ON 8 JUNE 1932
DUCKLING 393
DUCKLING 394
DUCKLING 395
DUCKLING 397
GROUP B: THESE DUCKLINGS HAD RECEIVED 0·5 C.C. DUCK EMBRYO ON 8 JUNE 1932
DUCKLING 398
DUCKLING 399
DUCKLING 400
DUCKLING 401
DUCKLING 402
GROUP C: THESE DUCKLINGS HAD RECEIVED NO PREVIOUS INOCULATIONS
DUCKLING 404
DUCKLING 405
DUCKLING 406

FIG. 1.

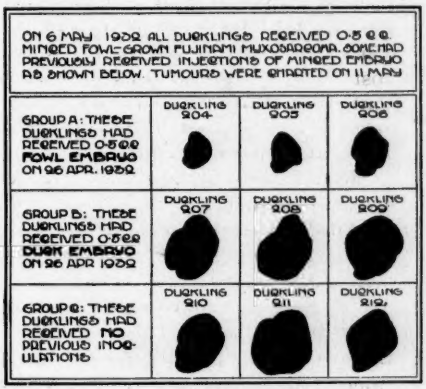
ON 6 MAY 1932 ALL DUCKLINGS RECEIVED 0·5 C.C. MINCED FOWL-GROWN FUJINAMI MYXOSARCOMA. SOME HAD PREVIOUSLY RECEIVED INJECTIONS OF MINCED EMBRYO AS SHOWN BELOW. TUMOURS WERE STARTED ON 11 MAY
GROUP A: THESE DUCKLINGS HAD RECEIVED 0·5 C.C. FOWL EMBRYO ON 26 APR. 1932
DUCKLING 204
DUCKLING 205
DUCKLING 206
GROUP B: THESE DUCKLINGS HAD RECEIVED 0·5 C.C. DUCK EMBRYO ON 26 APR 1932
DUCKLING 207
DUCKLING 208
DUCKLING 209
GROUP C: THESE DUCKLINGS HAD RECEIVED NO PREVIOUS INOCULATIONS
DUCKLING 210
DUCKLING 211
DUCKLING 212

FIG. 2.

FIG. 1.—Fowl-embryo completely failed to induce resistance to cell-free extract of fowl-grown Fujinami myxosarcoma.

FIG. 2.—Fowl-embryo induced definite though only partial resistance to minced fowl-grown Fujinami myxosarcoma.

advantage whether embryo tissue will similarly fail to prevent tumour formation when minced fowl-grown Fujinami tissue is used as the inoculum. This question has been posed in experiments, of which the following is an example:

On April 26th, 1932, 9 newly-hatched ducklings were divided into three groups. Straightway each duckling of Groups A and B was given 0·5 c.c. of minced embryo deep into the muscles of the right leg. Fowl embryo was used for Group A and duck embryo for Group B. Group C received no embryo. On May 6th, 10 days after the embryo injections, each duckling of all three groups received into the muscles of the left leg 0·5 c.c. of minced fowl-grown myxosarcoma. Fig. 2, which summarizes the results as they stood on May 11th, shows that in those ducklings which had received fowl-embryo, tumour formation had been resisted to a clearly recognizable but limited degree. At a somewhat later date all tumours of the experiment had become so nearly alike in size that differences could not be distinguished with certainty. This has

been a constant finding ; only at a relatively early stage in their growth do the tumours reveal the effects of embryo.

The results of the experiment show that when minced fowl-grown Fujinami myxosarcoma is injected into normal ducklings, the new tumours which arise have a two-fold origin. The minced tissue which is injected contains both malignant cells and infective tissue fluids. In the experiment the smallness of the new tumours which developed in ducklings which had received an injection of fowl embryo cannot be attributed to any resistance to the tumour-producing power of the infective fluids in the mince, since it has already been shown that embryo has no effect at all upon the tumour-producing power of cell-free extracts. The smallness of the tumours in these ducklings can only be attributed to a failure of the cells of the mince to become established, multiply, and play a normal *rôle* in the production of the new tumours. That an injection of fowl-embryo should have such an effect upon injected foreign cells is in accordance with what others have found in experiments upon tumours of mammals. But here a somewhat unexpected conclusion is reached for it follows that when, as in the experiment last described, minced fowl-grown Fujinami myxosarcoma is injected into normal ducklings, the resulting tumour, in its early stages at least, must consist of both duck and fowl cells. The tumour-cells derived from infected host-cells must of necessity be duck cells, and those derived directly from the injected fowl cells must of necessity be fowl cells. The direct experimental proof of the existence of both duck and fowl cells in these tumours has not been attempted, and evidence of this nature confirming the conclusions drawn from the experiments which have been described is wanting.

Presumably new tumours have a similar dual origin when the mince inoculated is derived from a Fujinami tumour which has been grown in a duck instead of a fowl, for it is to be anticipated that in ducklings duck cells will grow no less freely than fowl cells. Direct proof, however, has not been obtainable. So far as can be seen, ducklings which have received an injection of duck-embryo are no more resistant to an injection of minced duck-grown Fujinami myxosarcoma than are normal ducklings. Perhaps this was to be expected. Rous and Murphy (1914) record that in their experience with fowls the injection of hashed fowl-embryo does not confer resistance to minced fowl-grown Rous sarcoma No. 1. These results would be satisfactorily explained if, as one would anticipate, relatively slight resistance is induced when bird, embryo and tumour are all of one and the same species. In mice, the resistance induced by mouse-embryo is not really very strong. It is not sufficiently powerful to prevent the establishment of every strain of even the most susceptible of mouse tumours.

Since implanted embryo tissue—especially if it be of an alien species—grows less freely in adult animals than in those newly born, it seemed of interest to inquire whether when minced fowl-grown Fujinami myxosarcoma is injected into an adult duck the new tumour arises wholly, or only in part, by a process of infection of host-cells. The question was put in the following experiment :

On September 15th, 1932, 9 young adult ducks which ranged in weight between 640 and 960 grm. were separated into three closely similar groups on

the basis of their weights. Then each duck of Groups B and C received deep into the muscles of the right wing 0·5 c.c. of minced fowl-embryo. This was 14 days before the tumour mince was to be injected. The ducks of Group A received no embryo either at this time or later. On September 22nd, which was 7 days before the tumour injections were due, the ducks of Group C received a second injection of embryo; the dose was 0·5 c.c. as before; the site of injection was the left wing. On September 29th each of the 9 ducks received into the muscles in front of the left tibia-fibula 2·0 c.c. of minced fowl-grown Fujinami myxosarcoma. The results observed are shown in Fig. 3. October 5th was chosen for illustration, because on that day the tumours were of sizes which could be determined with sufficient accuracy, and because on that day retrogressive changes had not as yet greatly complicated the picture. As a matter of fact in two ducks, Nos. 144 and 147, retrogressive changes had actually set in during the previous 24 hours. For simplicity, however, the

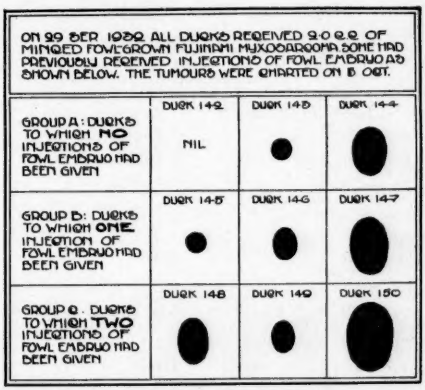
ON 29 SEP. 1920 ALL DUCKS RECEIVED 2·0 C.C. OF MINCED FOWLGROWN FUJINAMI MYXOSARCOMA SOME HAD PREVIOUSLY RECEIVED INJECTIONS OF FOWL EMBRYO AS SHOWN BELOW. THE TUMOURS WERE CHARTED ON 5 OCT.
GROUP A: DUCKS TO WHICH NO INJECTIONS OF FOWL EMBRYO HAD BEEN GIVEN
DUCK 142
DUCK 143
DUCK 144
NIL
GROUP B: DUCKS TO WHICH ONE INJECTION OF FOWL EMBRYO HAD BEEN GIVEN
DUCK 145
DUCK 146
DUCK 147
GROUP C: DUCKS TO WHICH TWO INJECTIONS OF FOWL EMBRYO HAD BEEN GIVEN
DUCK 148
DUCK 149
DUCK 150

FIG. 3.—In adult ducks fowl-embryo completely failed to induce resistance to minced fowl-grown Fujinami myxosarcoma.

tumours of these two ducks are represented in the chart as they would have been had they maintained their previous rate of growth, not as they actually were on October 5th.

It will be seen that in the experiment no evidence was obtained that fowl-embryo has any effect upon the formation of new tumours. This suggests that in normal adult ducks lineal descendants of inoculated cells play little or no part in the formation of new tumours. The experiment has not been repeated.

Actually the experiment just described was designed on the lines of one in which part-grown ducks—ducks two months old in fact—had been used. In this earlier experiment the ducks which received only one injection of embryo appeared to show a definite amount of resistance, whilst those which received two injections showed none at all.

But to return to ducklings. So far only experiments with the Fujinami tumour have been described. What of the Rous tumour? When the Fujinami myxosarcoma is transferred from fowls to ducklings by means of tumour

mince it is, as the experiments show, only in part by transplantation of tumour-cells; infection of host-cells also takes place. Can the same be said of the Rous sarcoma No. 1? Doubt is raised by the fact that whilst the Fujinami tumour is transferable by means of cell-free filtrates, the Rous sarcoma is not. The question of first importance, therefore, is whether a duckling which has received an injection of fowl embryo does or does not become completely resistant to the Rous sarcoma. The answer has been sought in many experiments, and it may be stated at once that a duckling which has received an injection of fowl-embryo does become completely resistant. The resistance is induced with ease, and tumours do not appear if the embryo has been injected

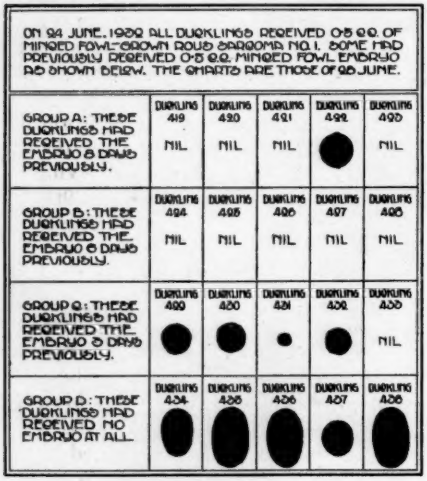
ON 24 JUNE, 1932 ALL DUCKLINGS RECEIVED 0·5 C.C. OF MINCED FOWL-GROWN ROUS SARCOMA NO. 1. SOME HAD PREVIOUSLY RECEIVED 0·5 C.C. MINCED FOWL EMBRYO AS SHOWN BELOW. THE CHARTS ARE THOSE OF 28 JUNE.
GROUP A: THESE DUCKLINGS HAD RECEIVED THE EMBRYO 8 DAYS PREVIOUSLY.
DUCKLING 419 NIL
DUCKLING 420 NIL
DUCKLING 421 NIL
DUCKLING 422
DUCKLING 423 NIL
GROUP B: THESE DUCKLINGS HAD RECEIVED THE EMBRYO 6 DAYS PREVIOUSLY.
DUCKLING 424 NIL
DUCKLING 425 NIL
DUCKLING 426 NIL
DUCKLING 427 NIL
DUCKLING 428 NIL
GROUP C: THESE DUCKLINGS HAD RECEIVED THE EMBRYO 8 DAYS PREVIOUSLY.
DUCKLING 429
DUCKLING 430
DUCKLING 431
DUCKLING 432
DUCKLING 433 NIL
GROUP D: THESE DUCKLINGS HAD RECEIVED NO EMBRYO AT ALL.
DUCKLING 434
DUCKLING 435
DUCKLING 436
DUCKLING 437
DUCKLING 438

FIG. 4.

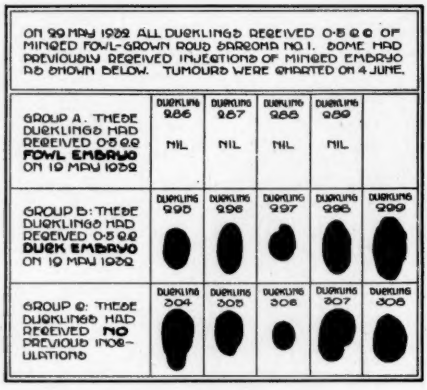
ON 20 MAY 1932 ALL DUCKLINGS RECEIVED 0·5 C.C. OF MINCED FOWL-GROWN ROUS SARCOMA NO. 1. SOME HAD PREVIOUSLY RECEIVED INJECTIONS OF MINCED EMBRYO AS SHOWN BELOW. TUMOURS WERE CHARTED ON 4 JUNE.
GROUP A. THESE DUCKLINGS HAD RECEIVED 0·5 C.C. FOWL EMBRYO ON 10 MAY 1932
DUCKLING 286 NIL
DUCKLING 287 NIL
DUCKLING 288 NIL
DUCKLING 289 NIL
GROUP B: THESE DUCKLINGS HAD RECEIVED 0·5 C.C. DUCK EMBRYO ON 10 MAY 1932
DUCKLING 295
DUCKLING 296
DUCKLING 297
DUCKLING 298
DUCKLING 299
GROUP C: THESE DUCKLINGS HAD RECEIVED NO PREVIOUS INOCULATIONS
DUCKLING 304
DUCKLING 305
DUCKLING 306
DUCKLING 307
DUCKLING 308

FIG. 5.

FIG. 4.—Complete failure of fowl-grown Rous sarcoma No. 1 to grow in ducklings which had received an injection of fowl-embryo 6 or 8 days before the test was made.
FIG. 5.—Duck-embryo completely failed to induce resistance to fowl-grown Rous sarcoma No. 1.

a week or so before the test is made. This finding is illustrated in the following experiment, which was so designed as to give an idea of how soon ducklings become resistant.

On June 16th, 1932, 8 days before they were to be injected with tumour tissue, 20 newly-hatched ducklings were divided into four groups, and then each duckling of Group A was inoculated with minced fowl-embryo. On the 18th, 6 days before the tumour injections were due, each duckling of Group B was similarly inoculated, and on the 21st, which was 3 days before the tumour injections, each duckling of Group C was inoculated. In each instance the dose of minced fowl-embryo was 0·5 c.c., injected deep into the muscles of the right leg. The ducklings of Group D received no embryo. On the 24th each duckling of the experiment received into the muscles of the left leg 0·5 c.c. of minced fowl-grown Rous sarcoma tissue. The results are shown in Fig. 4,

and from these it will be gathered that ducklings react in about the same time as mice. The resistance to implanted tumours reaches its maximum in mice in 8 or 10 days. In the experiment just described embryo induced an appreciable amount of resistance to the Rous sarcoma in 3 days and complete resistance in 6 days. One duckling alone, No. 422, gave an anomalous result. In this duckling, however, the embryo tissue had grown less freely than in any other duckling of the experiment, which suggests that possibly a material portion of the injected embryo tissue may have run back along the needle-track and escaped unobserved. In the following experiment the efficacy of duck embryo was compared with that of fowl.

On May 19th, 1932, 14 newly-hatched ducklings were divided into three groups. Then straightway each duckling of Group A was given 0·5 c.c. of minced fowl embryo and each duckling of Group B was given 0·5 c.c. of minced duck-embryo, each injection being made into the muscles of the right leg. The ducklings of Group C received no embryo at all. The embryo cells —both fowl and duck—behaved in a normal way. They grew for a time and formed palpable masses of tissue, then presently these masses began to disappear. Embryo tissue was still present, however, and apparently still living when the experiment was brought to an end on June 4th, 1932. On May 29th, 10 days after the injections of minced embryo had been given, all the ducklings of the experiment received into the left leg 0·5 c.c. of mince prepared from a Rous sarcoma No. 1, which had been grown in a fowl. The birds used and the results of the tumour injections are shown in Fig. 5.

It will be seen that no tumours grew in the ducklings which had received an injection of fowl embryo, whilst in those which had received duck embryo, tumours appeared just as readily as in the normal ducklings. It will have been observed that duck embryo similarly failed to protect against cells of fowl-grown Fujinami myxosarcoma in the experiment which has been summarized in Fig. 2. Apparently then, in ducklings as in mice, the resistance induced by embryo tissue is effective only when embryo and tumour are taken from the same species of animal. Clearly the action of embryo in ducklings is essentially similar to the action in mice, and even if the direct information gained in experiments with Fujinami's tumour were not available, these experiments with the Rous sarcoma No. 1 could be interpreted with confidence in the light of what is known to occur in the experiments which others have made with mammalian tumours. The experiments with the Rous tumour show that, unlike the Fujinami myxosarcoma, the Rous sarcoma No. 1 grows in ducklings purely as a transplantable tumour, and that the cells of a daughter-tumour are all lineal descendants of cells of the inoculum. Since the resistance produced by embryo is complete, it means that duck cells are not infected by the Rous agent even when that agent is presented as efficiently as it must be when tissue mince is used. Thus the significance of the constant failure to produce tumours in ducklings by means of filtrates of the Rous sarcoma becomes clear. Filtrates of the Fujinami tumour, as already pointed out, produce tumours readily in ducklings, and correlated with this is the finding that in ducklings a portion of the tumour produced by minced fowl-grown Fujinami tissue is derived from host-cells. If the experiments made with the Rous sarcoma will bear the interpretation which has been placed upon them, as seems beyond

doubt, it follows that when a Rous sarcoma No. 1 is propagated in ducklings —no matter for how many generations—the tumour-cells must remain fowl cells, since they are lineal descendants of the fowl-tumour cells injected into the first duck of the series. In fact it means that the propagation of the Rous sarcoma in ducklings is exactly comparable with Murphy's (1912) propagation of a mouse tumour in chick embryo, and is closely similar to the propagation in mice of a mouse tumour like carcinoma No. 63 of the Imperial Cancer Research Fund. If this propagation of the Rous tumour in ducklings is actually of the nature which has been inferred from the experiments, then one would expect that a *duck*-grown Rous sarcoma would always fail to grow in ducklings which had received an injection of fowl embryo, just as fowl embryo itself fails to grow, but would grow normally in ducks which had received duck embryo. The fact that filtrate of a duck-grown Rous sarcoma will never infect ducklings is suggestive, but the actual attempt to produce a tumour by means of duck-grown Rous sarcoma injected into a duckling which has already been inoculated with fowl embryo, though desirable, has not been made. The results of tests made with a similar object but along other lines will be described in a subsequent paper.

SUMMARY AND CONCLUSIONS.

It is generally accepted that the resistance induced in mammals by an injection of normal tissue is solely directed against the establishment of transplantable foreign cells. The power of embryo to induce such a resistance has been used in a study of the process by which the Rous sarcoma No. 1 and the Fujinami myxosarcoma may be transferred from fowls to ducks.

Experiment shows that in ducks fowl-embryo fails to induce resistance to the infective agent of a fowl-grown Fujinami myxosarcoma. This supports and confirms the conclusions drawn from the study of mammalian tumours, namely, that the resistance induced by embryo is not directed against any quality peculiar to malignant tumours.

Experiments show that ducklings which have received an injection of minced fowl-embryo do resist to some extent the formation of a new tumour by means of minced fowl-grown Fujinami tumour, and from what has been said already it is clear that the resistance can only be to cells of the inoculum which, normally, would contribute directly to the formation of the new tumour.

From the two kinds of experiment mentioned above, it is concluded that in normal ducklings the new tumours which follow upon the injection of minced Fujinami tissue are formed in part by multiplication of infected host-cells, and in part by multiplication of cells of the inoculum. In other words, it is concluded that the process is dual—partly an infection and partly a transplantation.

Since the Rous sarcoma No. 1 cannot be transferred to ducklings by filtrates, and since the production of tumours by means of minced fowl-grown Rous sarcoma tissue is completely inhibited in ducklings which have been inoculated with fowl-embryo, it is concluded that in ducklings the Rous sarcoma grows purely as a transplantable tumour, all cells of a new tumour being lineal descendants of cells of the inoculum.

This difference between the Rous and Fujinami tumours, the agent of one being able to infect duck cells and the other not, provides the explanation of differences in the conditions which have been found necessary for successful transfer of the tumours from fowls to ducks, and from one duck to another.

The results of the experiments with the Rous and Fujinami tumours emphasize once again the necessity of distinguishing sharply between transplanted and spontaneous tumours, and the danger of assuming that conditions which affect a transplanted tumour will similarly affect spontaneous tumours. They emphasize, too, that even in experiments with "filterable" tumours it is unsafe to assume lightly that transfer by means of minced tissue is the exact equivalent of transfer by means of cell-free extracts.

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ON SERUM BILIRUBIN: II. THE RELATIONSHIP BETWEEN THE BILIRUBIN CONTENT OF SERUM FROM DIFFERENT TYPES OF JAUNDICE AND THE ICTERUS INDEX OF THE SERUM AFTER REMOVAL OF PROTEINS.

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IN a previous communication (White, 1932), it was shown that by using a modification of the Thannhauser and Andersen technique, the bilirubin content of icteric serum could be determined with reasonable accuracy. Jaundice is not, however, a clinical entity, and although it is at all times desirable to be able to determine the actual content of any blood constituent the concentration of which varies in abnormal conditions, such an estimation can only convey to the clinician an indication of the extent of the jaundice, and cannot furnish any information of the nature of the disease of which jaundice is the visible symptom. In 1916 van den Bergh and Müller suggested that by testing icteric sera with Ehrlich's diazo reagent, a means existed of differentiating

between jaundice of hæmolytic origin and that of the obstructive type. This test, later elaborated by van den Bergh (1921) and McNee (1922, 1923), and known as the "Van den Bergh" test, was based on the observation that serum from cases of hæmolytic jaundice reacted with the diazo reagent slowly, or only after addition of alcohol (the *delayed* or *indirect* reaction), while in the case of "obstructive" serum the reaction was immediate and maximal (the *immediate* or *direct* reaction). The deduction followed that in icteric serum the bilirubin could exist in two different modifications, which were referred to as *direct reaction* and *indirect reaction* types. Although this test has been, and still is, widely used, it has been subject to criticism not only from the standpoint of clinical interpretation (Blankenhorn, 1927 ; Mogena, 1929 ; Nicholson, 1930), but also with regard to the reaction itself. Hubbard and Allison (1929) and Snider and Reinhold (1930) have reported that the rate of the diazo reaction depends mainly upon the concentration of bilirubin in the serum, an immediate reaction being obtained when the bilirubin content is high, and conversely a delayed type of reaction being observed with a low concentration. Further, Müller and Engel (1931) examined the absorption spectra of sera treated with the diazo reagent, and could not find evidence of the existence of more than the one pigment. These criticisms, whilst casting doubt upno the validity of the diazo reaction as a means of differentiation, do not, however, disprove the theory of the dual nature of bilirubin in icteric serum.

Van den Bergh and Müller, amongst other observations, reported that in cases of obstructive jaundice when the serum proteins were precipitated by alcohol, the precipitate was generally highly coloured, suggesting adsorption of pigment, whereas in cases of hæmolytic jaundice the protein precipitate was invariably colourless. This finding has been confirmed by Thannhauser and Andersen (1921), Barron (1931) and others (including the writer), and consequently the assumption can be made that when alcohol is added to serum, the bilirubin responsible for the indirect reaction, not being adsorbed upon the protein precipitate, will remain dissolved in, and will pigment, the aqueous alcoholic liquid. If only this type of pigment is present in the serum, it should be possible to establish a relationship between the colour intensity in the alcoholic extract and the bilirubin content of the serum. This was attempted by adding varying quantities of bilirubin to serum which had been rendered free from the pigment (see White, 1932), and comparing the known bilirubin concentrations with the colour indices of the respective alcoholic extracts. The procedure for determining the colour index was essentially the same as that in general use for determining the icterus index of serum (Maue, 1922), and was carried out as follows : A measured volume of serum was treated with twice its volume of 95 per cent. alcohol in a centrifuge tube, mixed, allowed to stand for 10 minutes, and centrifuged. The supernatant liquid was compared in a colorimeter against the usual icterus index standard (0·01 per cent. potassium dichromate solution), the dilution being taken as 20/7 to allow for the contraction due to alcohol (van den Bergh). If necessary a measured quantity of this alcoholic liquid was further diluted with a known volume of a 2 : 1 alcohol-water mixture before comparison, and the index calculated from the formula :

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{20}{7} \times \frac{\text{Final volume}}{\text{Volume supernatant liquid taken}} = \text{Index.}$$

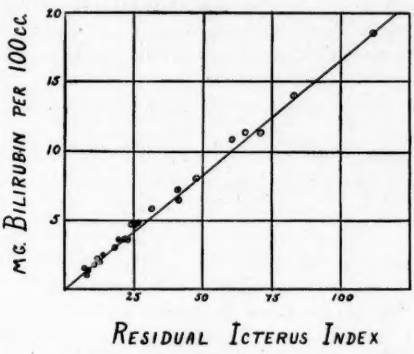

FIG. 1.—Graph showing the relationship between the residual icterus index and the bilirubin content of artificially icteric serum.

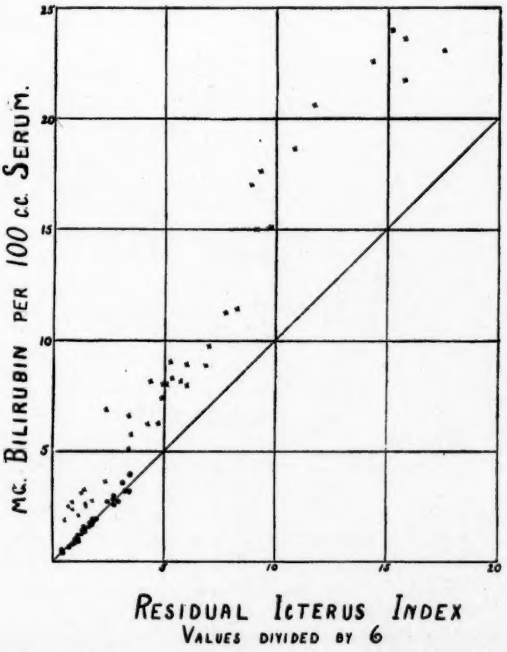

FIG. 2.—The bilirubin content of normal and icteric serum compared with the corresponding residual icterus index values divided by 6. ⊙ = normal cases; ● = jaundice cases of hæmolytic origin; × = jaundice cases of other than hæmolytic origin.

Since the same standard is used and allowance made for the initial dilution of the serum with alcohol, the resultant value is directly comparable with the icterus index. The icterus index is a figure which indicates the degree of yellow pigmentation in the serum, and since this second value represents the serum pigment remaining in solution after removal of proteins, it can be regarded as the *residual icterus index*.

On plotting the values thus obtained against the known bilirubin content of the serum the graph shown in Fig. 1 was obtained, and it will be at once apparent that under the conditions of the experiment the residual icterus index is directly proportional to the concentration of bilirubin in mgm. per 100 c.c. serum, the ratio being approximately 6 to 1. Although the amount of added bilirubin varied from 1 to 18·5 mgm. per 100 c.c. the regularity of the graph suggests that there can have been little, if any, adsorption of pigment upon the precipitated proteins.

A series of cases of clinical jaundice* was then investigated ; in each case the residual icterus index was estimated as described, and the serum bilirubin determined according to the writer's modification of the Thannhauser and Andersen technique. The residual icterus index was divided by 6, and the resultant values plotted against the serum bilirubin in mgm. per 100 c.c. The results are shown in Fig. 2, and comprise values from normal as well as jaundice cases.

DISCUSSION.

In Fig. 2 it will be noticed that the points on or close to the line bisecting the right angle are all from normal cases or jaundice cases of hæmolytic origin ; that is, in these cases the residual icterus index, when divided by 6, gives a value which is approximately equal to the bilirubin content in mgm. per 100 c.c. The other points plotted represent jaundice cases of other than hæmolytic origin. These all lie well above the line of direct proportionality, indicating that in each of these cases the residual icterus index, divided by 6, gives a value which is less than the bilirubin content of the serum in mgm. per 100 c.c. Since this index is directly proportional to the amount of serum bilirubin remaining in solution when the proteins are precipitated by alcohol (Fig. 1), it follows that in these cases only part of the total bilirubin is in solution, or in other words, some pigment has been thrown down with the precipitate. From these results it appears that there is a definite relationship between the residual icterus index and the serum bilirubin content in all cases where the latter is directly due to the normal or abnormal breakdown of hæmoglobin, and that this relationship does not hold for jaundice cases of other than hæmolytic origin. The cases in the latter category included both obstructive and toxic types of jaundice, but no differentiation was observed between them ; in all cases in this category there appeared to be some factor which partially inhibited the complete solution of the pigment in the alcoholic extract. Since

* Included amongst the normal cases are three whose bilirubin content was 3·2, 3·0 and 1·7 mgm. per 100 c.c. serum ; the respective icterus indices were 19·1, 15·7 and 9·4. Although these are definitely abnormal values, those three individuals were in normal health, and had no previous history of illness to account for the high values obtained. They are, therefore, included in the normal category, although they are possibly congenital anomalies.

this factor is not present in normal or "hæmolytic" serum, it represents a change either in the bilirubin molecule or in its combination with other constituents of the serum, and is independent of the concentration of the pigment (see Fig. 2).

THE "ICTERIC RATIO".

The object of this investigation was an endeavour to obtain a simple test, suitable for routine work, which would prove of assistance in the differential diagnosis of jaundice. To determine the relationship between the serum bilirubin and the residual icterus index, a quantitative bilirubin estimation is essential, and although the technique is not difficult, it is somewhat time-consuming, and from the clinical standpoint, unless the actual bilirubin content is required, unnecessary. The same result can be obtained in a much simpler fashion. Where the serum bilirubin is entirely composed of the type which is formed from the normal or abnormal breakdown of hæmoglobin, this pigment will be extractable by alcohol. Consequently there should be the same degree of bile pigmentation in the serum deproteinized by alcohol (when allowance is made for the dilution) as in the original serum ; or in other words, the icterus index, whether determined before or after removal of the proteins, should have substantially the same value, and the ratio of the one to the other should be 1. This *icteric ratio* can therefore be represented as $\frac{\text{icterus index}}{\text{residual icterus index}}$, and that it approximately corresponds to the relationship between the bilirubin content and the residual icterus index divided by 6 is shown by the results reported in Table I.

TABLE I.—*The Icteric Ratios of Sera from some Different Types of Clinical Jaundice.*

Case No.	Diagnosis stated.	(*a*) Bilirubin content, mgm. per 100 c.c.	(*b*) Icterus index.	(*c*) Residual icterus index.	Icteric ratio $\frac{(b)}{(c)}$	$\frac{(a)}{(c) \times 1/6}$.
48	Bronchial pneumonia	2·8	16·1	11·1	1·45	1·5
23	Catarrhal jaundice	2·1	14·2	7·0	2·00	1·9
53	,, ,,	3·1	21·7	8·0	2·70	2·4
71	,, ,,	3·6	23·2	14·3	1·60	1·8
32	Chr. cholecystitis (stones)	2·5	15·0	9·2	1·60	1·7
74	Chr. cholecystitis (stones)	2·4	16·6	5·6	3·00	2·7
72	Stricture, common duct	2·8	16·4	5·3	3·10	3·1
60	Pernicious anæmia	2·9	17·4	17·5	1·00	1·0
86	,, ,,	2·9	16·7	16·7	1·00	1·0
54	Polycythæmia (phenyl hydrazine administered)	4·0	21·1	21·1	1·00	1·1

These results are a few typical examples obtained from cases in which the icterus indices were about the same value. A possible source of error in the determination of this ratio lies in the fact that the yellow pigment, carotene, is also present in serum. It was shown (White, 1931) that in 26 non-diabetic cases (both normal and pathological) the greatest amount of serum colour found due to carotene could be represented by an icterus index of 1·4, while the mean value was in the neighbourhood of 1. A factor of this magnitude can have but little effect upon the ratio when the icterus index is large, but the smaller the icterus index, the greater will be the probable error. In practice it has been found that with an icterus index of 10 or less a $\pm$ 20 per cent. variation in the ratio is without significance.

TABLE II.—*Summary of Results obtained with Normal and Icteric Sera.*

Icteric ratio	Number of cases.		
	Normal.	Jaundice of hæmolytic origin.	Jaundice of non-hæmolytic origin.
0·9–1·0	7	4	..
1·0–1·1	6	10	..
1·1–1·2	..	3	..
1·2–1·3	..	..	..
1·3–1·4	..	..	5
1·4–1·6	..	..	13
1·6–1·8	..	..	13
1·8–2·0	..	..	5
2·0–2·5	..	..	10
2·5–3·0	..	..	2
3·0–4·0	..	..	9
Total	13	17	57

The icteric ratios determined on some 80 cases are summarized in Table II, and it will be noticed that whereas in normal cases and jaundice cases of hæmolytic origin the ratio as a rule varied from 0·9 to 1·1, and was in no instance greater than 1·2, with other types of jaundice the ratio varied mainly from 1·3 to 1·8, but was much higher in a considerable proportion of the cases. It seems possible, therefore, that this icteric ratio may prove of assistance in the diagnosis, particularly, of cases of latent or slight jaundice. It has the advantage that it can be determined upon 2 c.c. of serum in a few minutes.

NORMAL SERUM BILIRUBIN VALUES.

The values recorded in the literature for normal serum bilirubin vary considerably according to the method of estimation used. Barron (1931) has tabulated a series of " normal " values as reported by different workers, and they range all the way from 0·1–0·3 mgm. per 100 c.c. (van den Bergh, 1918) to 0·5–3·5 mgm. per 100 c.c. (Perkin, 1927). Barron himself considers the normal

range to be 0·1–0·5 mgm. per 100 c.c. During this investigation the serum bilirubin values of 12 normal individuals were determined. Each of these persons was in good health, and had an icterus index within normal limits. The bilirubin was estimated by the modified Thannhauser and Andersen technique described in Part I, which has been shown to be of fair accuracy (White, 1932). The values ranged from 0·4 to 1·4 mgm., with a mean value of 0·8 mgm. per 100 c.c. This is of interest since Müller and Engel (1930), on the basis of their spectrophotometrical measurements, claimed that normal values as generally accepted were much too low. They considered the normal range to be from 0·6 to 2·0 mgm. per 100 c.c. Although 12 cases is much too small a number from which to draw definite conclusions, it appears very probable that the low values hitherto accepted have been due to the inaccuracy of the methods used in the determinations, and that the normal range is at least of the order of 0·5–1·0 mgm. and quite possibly 0·5–1·5 mgm. per 100 c.c. of serum.

SUMMARY.

The *residual icterus index* is the name given to the icterus index when determined on serum from which the proteins have been removed by precipitation with alcohol.

It has been shown that in serum artificially rendered icteric by the addition of bilirubin, the *residual icterus index* is directly proportional to the bilirubin content in mgm. per 100 c.c., the ratio being approximately 6 to 1.

Results have been obtained indicating that within the limits of this investigation, this relationship also holds for the serum of normal individuals and of jaundice cases of hæmolytic origin, but does not hold for other types of jaundice.

The *icteric ratio* of serum is defined as the ratio of the *icterus index* to the *residual icterus index*.

In normal serum and icteric serum of hæmolytic origin this *icteric ratio* has been found to be generally less than 1·2, whereas in all other jaundice cases investigated it was almost invariably much higher. Since this ratio is easily and quickly determined, it is suggested that in cases of latent or slight jaundice it might prove of assistance in differentiating such cases into " hæmolytic " and " non-hæmolytic " categories. A further differentiation of the latter category into " obstructive " and " toxic " types has not been found possible by this method.

It is claimed that the generally accepted normal values for serum bilirubin are too low, and that the normal range is at least 0·5 to 1·0 mgm. and possibly 0·5 to 1·5 mgm. per 100 c.c. serum.

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MODERN TESTS OF LIVER EFFICIENCY APPLIED TO EXPERIMENTAL SHALE OIL LIVER NECROSIS.

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LIVER necrosis in rabbits, the result of intra-peritoneal administration of shale oil, was described in 1931 (Polson). This lesion was readily produced, and appeared suited to the investigation of liver dysfunction. The present attitude towards tests of liver efficiency was summarized by Fowweather (1929) when he said that " it is extremely difficult to assess the true value of most of the methods in use. . . . With regard to most of them very conflicting results have been reported by various workers ". The present research concerned the application of seven of the modern tests to rabbits which had acute liver necrosis, following the intra-peritoneal administration of shale oil, to determine which, if any, of these tests provided evidence concerning the functional disturbance in this experimental lesion.

EXPERIMENTAL PROCEDURE.

The forty rabbits used had an average weight of approximately 2 kgm., and there were about equal numbers of males and females. Natural disease

was absent from all. Five young rabbits, 3½–4 months old, formed a subsidiary group of experiments concerned with changes in the amino-acid concentration of the blood. Acute liver necrosis was produced by the intra-peritoneal administration of 10 c.c. for adult and 6–8 c.c. for young rabbits of the lubricating oil, or the lubricating oil plus solid paraffin fractions of shale oil. In the preliminary experiments the rabbits died on the fourth to fifth days of experiment; in later experiments the rabbits were killed by throat-cut on the third or fourth days, at which time severe liver damage was present. A post-mortem examination was at once made, and portions of liver and kidney were submitted to histological examination.

Six rabbits were subjected to the phenoltetrachlorphthalein test as described by Rosenthal (1922). Three others had doses of 10 c.c. of a 1 per cent. aqueous solution of rose bengal intravenously, and blood samples were taken sixteen minutes and one hour afterwards. A third group of six rabbits was used to test the Van den Bergh reaction as described by McNee (1923). A fourth group of nine rabbits provided evidence concerning changes in the urea concentration of the blood as determined by the method of Fowweather (1925). Changes in the amino-acid concentration of the blood were observed in a fifth group of ten adult and five young rabbits. The method of estimation was that of Folin (1922). The lævulose tolerance test was applied to seven rabbits. Of these three received lævulose (A. R., British Drug Houses, Ltd.) intravenously in doses of 2 c.c. of a 50 per cent. aqueous solution. Blood samples were taken before and at half-hourly intervals until two hours afterwards; the sugar concentration in the samples was determined by the method of Maclean (1924). The other four rabbits received lævulose intra-peritoneally in doses of 10 c.c. of a 50 per cent. solution; blood samples were taken before and at hourly intervals until three hours afterwards. The galactose tolerance test was similarly applied to ten rabbits, of which five received galactose (A. R., B. D. H. Ltd.) intravenously in doses of 5 c.c. of a 20 per cent. aqueous solution; the sugar was administered to the other five by the intraperitoneal route in doses of 10 c.c. of a 50 per cent. solution.

All of these tests were applied to the rabbits when healthy, and at daily intervals after the administration of shale oil. Blood samples were taken at 10 a.m. to 10.30 a.m. before food was given.

EXPERIMENTAL OBSERVATIONS.

As far as it was possible to judge by macroscopic and microscopic examination the liver damage in each of the rabbits was of similar severity, and such individual differences as were found were considered to have no serious influence on the validity of the results obtained in the groups of experiments, since each was composed of several rabbits.

1. *The phenoltetrachlorphthalein test.*—In a negative test all the dye administered should have disappeared from the serum at the end of an hour. Six rabbits were tested prior to experiment. Although the dosage was calculated on the weights of the rabbits, much dye was present in the sera of three rabbits, and a faint trace persisted in the other three at the end of an hour after administration. The latter were given shale oil, and on the second and

fourth days of experiment much dye was present in their sera at the end of an hour. The value of this result was mitigated by the " positive " results obtained prior to experiment. Moreover, accurate estimation of the amount of dye present was precluded at an early stage in rabbits which had acute liver necrosis owing to the development of an opalescence in the serum.

2. *The rose bengal test.*—The test was applied on two occasions to three rabbits when healthy. On the first the amount of dye present in the serum was 71, 83 and 40 per cent. respectively sixteen minutes after administration. On the second occasion the amounts were 40, 42 and 28 per cent. at sixteen minutes, and 23, 21 and 21 per cent. at the end of an hour. On the first day of experiment the sixteen-minute samples contained 84, 95 and 77 per cent. of the dye, while the corresponding results at an hour were 45, 56 and 31 per cent. Thereafter accurate estimation was precluded by an opalescence of increasing density in the blood-serum.

3. *The Van den Bergh reaction.*—Completely negative results were obtained from all six rabbits when healthy and throughout the experiments, which were concluded by the death of the rabbits on the fourth or fifth days. The negative results were expected, since jaundice was absent from these rabbits as from all others which had acute liver necrosis. If the liver plays an important part in the production and circulation of the bile in rabbits, it is indeed remarkable that jaundice was absent in these experimental animals.

TABLE I.—*The Blood-Urea Content in Acute Liver Necrosis in mgm./100 c.c.*

Rabbit.	Healthy content.	Day 1.	Day 2.	Day 3.	Day 4.	Day 5.	Remarks.
440	57	66	43	45	88	..	Died at noon on Day 4.
441	49	47	47	64	96	225	Ill and killed on Day 5.
442	57	68	55	64	137	..	Died at noon on Day 4.
443	49	75	49	55	60	..	Found dead at 10 a.m. on Day 5.
444	45	42	68	49	71	..	Died on Day 4.
445	55	..	73	81	83	..	Found dead at 10 a.m. on Day 5.
534	47	72	85	107	112	..	Killed on Day 4.
538	56	72	..	192	320	..	,, ,,
539	49	68	55	88	168	..	,, ,,
Averaged results	52	64	59	83	126	225	..

4. *Changes in the urea concentration of the blood.*—The healthy fasting concentration in nine rabbits ranged from 47 mgm./100 c.c. to 57 mgm./100 c.c. The details of experiment are shown in Table I.

A progressive rise in urea concentration was observed in rabbits 445, 534, 538 and, less apparent, in 441, in which the increase was delayed until the third day. This rabbit survived until the fifth day of experiment, when its urea concentration was 225 mgm. A daily fluctuation was observed in five of the rabbits, as for example in rabbits 443 and 539. On the fourth day of experiment the urea concentration exceeded healthy limits in all rabbits. The averaged results of this group of experiments showed that no appreciable rise in the blood-urea concentration occurred until the third day of experiment.

While there was an increase in urea concentration, and in several rabbits this was progressive, it is not permissible to assume that these changes were indicative of liver damage, nor were they a measure of its severity. For, in another series of experiments, the subject of a separate communication, it was evident that renal dysfunction was in part responsible for the increase in urea concentration in the blood.

TABLE II.—*Changes in the Amino-Acid Nitrogen Concentration of the Blood in Acute Liver Necrosis in mgm./100 c.c.*

Rabbit.	Healthy concentration.	Day 1.	Day 2.	Day 3.	Day 4.	Day 5.	Remarks.
440	7·35	6·37	7·00	7·80	11·70	..	Died on Day 4.
441	7·70	5·10	7·39	8·75	14·70	28·0	Killed when ill on Day 5.
442	9·10	6·10	6·37	..	15·56	..	Died on Day 4.
443	8·75	7·00	7·00	9·34	11·70	..	Found dead at 10 a.m. on Day 5.
444	8·40	6·37	7·00	7·78	12·73	..	Died on Day 4.
445	7·49	5·10	6·10	7·00	9·34	..	Found dead at 10 a.m. on Day 5.
488	8·90	7·37	7·80	7·80	11·67	..	Killed on Day 4.
534	9·46	9·52	9·93	10·77	10·76	..	,, ,,
538	9·88	9·80	9·72	15·72	19·00	..	,, ,,
539	10·39	9·52	9·52	10·45	14·89	..	,, ,,
Averaged results	8·74	7·23	7·78	9·49	13·21	28·0	..

5. *Changes in the amino-acid nitrogen concentration of the blood.*—The fasting concentration in 28 adult rabbits ranged between 6·76 and 10·39 mgm., and averaged 8·38 mgm./100 c.c. These values correspond closely with those of Okada and Hayashi (1922), who applied the method of Okada (1918) to 35 blood samples. They found the minimum concentration was 7·22 mgm., the maximum 10·60 mgm., and the average 9·20 mgm. 100/c.c.

Prior to experiment the amino-acid content of the ten rabbits given oil ranged from 7·35 to 10·39 mgm., and averaged 8·74 mgm/100 c.c. It was not until the third day of experiment (Table II) that increase in amino-acid concentration was observed. On that day 538, previously yielding results within healthy limits, showed an abrupt rise in amino-acid concentration from 9·72 to 15·72 mgm./100 c.c. The concentration in 534 and 539 was just beyond healthy limits, being 10·77 and 10·45 mgm. respectively. Five of the other six rabbits continued to yield results within healthy limits. On the fourth day of experiment a rise, in most instances abrupt, occurred in the amino-acid concentration of all the rabbits, with the exception of 445. Although on this day its amino-acid concentration was 9·34 mgm., there had been a rise by 2·34 mgm. above that of the previous day.

The averaged results of the ten rabbits given oil showed that the amino-acid concentration of the blood remained within healthy limits until the fourth day of experiment, when an abrupt rise occurred. One observation was made on the fifth day on rabbit 441, which had an amino-acid concentration of 28·0 mgm./100 c.c.—nearly double the amount present on the previous day.

Another feature of these results was the fall in amino-acid content observed in the majority of the rabbits on the first day of experiment. This fall was

considerable, for it exceeded 1·0 mgm. in six rabbits, and the lowered concentration was maintained on the second day in four of the rabbits. The significance of this observation is not clear, but it serves to demonstrate that increase in amino-acid concentration is not to be ascribed to the constituents of the oil administered.

The foregoing results were confirmed by experiments on five young rabbits aged 3½–4 months. Their average fasting concentration when healthy was 9·09 mgm. A fall to 7·96 mgm. was observed on the first day, on the second the average concentration was 8·49 mgm., and there was an abrupt rise to 11·73 mgm. on the third day of experiment.

Although it was probable that increase in the amino-acid concentration was influenced by deficient renal excretion, it was, nevertheless, a better indication of liver damage than was increase in urea concentration. Rabbits which had an amino-acid concentration exceeding 12·00 mgm. were invariably found to have liver necrosis at death. Occasionally such damage was found in rabbits in the blood of which the amino-acid concentration was within healthy limits. This is not surprising, since there was no appreciable rise in the blood amino-acid on the second, and in most not until the fourth day of experiment, yet rabbits killed on the second and third days had severe liver damage.

These experiments showed that there was a reserve in the liver abolished only by gross damage. Once that reserve was destroyed the amino-acid concentration rose to extreme limits, as seen on the last day of experiment. The test, therefore, is of limited value, but when positive is of grave prognostic significance.

6. *The lævulose tolerance test.*—The results of duplicated estimations of the blood-sugar content of the three rabbits which received lævulose intravenously are shown in Table III.

When interpreting the results of this test on human subjects, Fowweather (personal communication, 1932) considers a maximum sugar concentration exceeding 140 mgm. or a maximum rise exceeding 30 mgm. indicative of liver damage. These are not rigid criteria and may be modified in individual cases. In the present experiments these criteria, are used, together with a third, namely, failure to return to the fasting level at the end of two hours. Prior to experiment the rabbits were healthy as judged by these criteria.

The raised fasting blood-sugar content of 532A and 529A on the first day of experiment may have been due to emotional disturbance, the influence of which upon the blood-sugar content was demonstrated by Folin and Berglund (1922). The results obtained on the second day were equivocal, and did not admit the diagnosis of liver dysfunction. Those of the third day were irregular. One rabbit (532A) yielded a positive result, while the test on 529A was completely negative. Yet the latter had severe liver damage when killed twenty-four hours later, and control rabbits killed on the third day had unmistakable liver damage. The two surviving rabbits yielded completely positive results on the fourth day of experiment.

The following are the averaged results of those tests in which lævulose was administered intraperitoneally to four rabbits : When healthy the fasting concentration was 126 mgm., the maximum concentration 357 mgm., and that at the end of three hours was 156 mgm. Corresponding results on the

TABLE III.—*Lævulose Tolerance Tests in Acute Liver Necrosis. Dosage of Lævulose = 2 c.c. of 50% Intravenously.*

Rabbit.	Fasting concentration in mgm./100 c.c.	Blood-sugar concentration after dose of lævulose. ½ hour.	1 hour.	1½ hours.	2 hours.	Maximum rise in mgm.	Increase or decrease at 2 hours in mgm.
(*a*) When healthy :							
532A	118	118	125	125	100	+6	−19
	125	122	131	131	106		
529A	118	118	100	112	112	±0	−3
	116	118	98	112	116		
528A	112	137	112	106	112	+25	+3
	106	131	112	112	112		
(*b*) First day of experiment :							
532A	180	118	81	81	70	−63	−107
	180	116	81	81	75		
529A	175	131	116	93	100	−38	−72
	168	137	118	100	100		
528A	100	134	137	93	86	+30	−13
	106	131	134	98	93		
(*c*) Second day of experiment :							
532A	168	175	168	131	112	+10	−52
	168	181	175	140	120		
529A	162	193	118	168	152	+25	−5
	168	186	116	168	168		
528A	168	162	175	126	156	±0	−14
	175	168	168	131	160		
(*d*) Third day of experiment :							
532A	125	156	138	162	170	+48	+48
	125	162	138	172	175		
529A	127	128	136	125	125	+9	−2
	..	136	..	131	125		
528A	93	100	137	93	93	+44	−3
	98	110	143	93	93		
(*e*) Fourth day of experiment :							
529A	93	184	135	120	118	+91	+26
	98	190	135	125	125		
528A	100	186	144	135	131	+88	+33
	100	190	150	140	135		

second day of experiment were 116, 245 and 135 mgm., those for the third day were 87, 277 and 253 mgm., and those for the fourth day were 106, 383 and 275 mgm. In this series it appeared that the criterion of liver damage was a high sugar concentration at the end of three hours, and judged by this, a positive result was obtained on the third day of experiment.

With this test a clearly positive result was indicative of the liver damage which was invariably found at autopsy ; but not all rabbits which had severe liver damage yielded a positive result, as seen in Rabbit 529A.

TABLE IV.—*Galactose Tolerance Tests in Acute Liver Necrosis. Dose of Galactose = 5 c.c. of 20% Intravenously.*

Rabbit.	Fasting concentration in mgm./100 c.c.	Blood-sugar concentration after dose of galactose. ½ hour.	1 hour.	1½ hours.	2 hours.	Maximum rise in mgm.	Increase or decrease at 2 hours in mgm.
(*a*) When healthy:							
531A	130	143	100	100	98	+12	−26
	125	137	100	93	106		
533A	131	181	131	118	98	+53	−35
	131	186	140	125	93		
535	125	175	150	112	112	+50	−10
	118	168	156	116	112		
522	156	212	181	181	156	+56	0
536	116	160	143	137	116	+44	0
(*b*) Day 1 of experiment:							
531A	147	154	135	112	100	+12	−46
	143	160	137	118	98		
533A	118	133	143	118	112	+20	−8
	125	133	138	120	116		
535	116	156	135	93	112	+36	−2
	118	150	140	93	118		
522	143	186	181	156	125	+48	−18
	137	190	181	150	118		
536	118	157	131	118	116	+39	−2
(*c*) Day 2 of experiment:							
531A	135	200	193	168	125	+68	−10
	135	206	186	178	125		
533A	206	275	281	246	206	+78	+2
	206	268	287	240	209		
535	143	168	150	118	143	+28	+2
	137	168	156	125	140		
522	175	231	212	181	193	+56	+15
	181	236	218	186	193		
536	137	182	160	137	120	+45	−17
(*d*) Day 3 of experiment:							
531A	143	256	223	186	168	+106	+19
	150	250	225	181	164		
533A	156	250	225	200	162	+94	+5
	156	..	230	198	160		
535	131	143	137	131	112	+13	−24
	137	150	143	135	108		
522	162	250	256	225	218	+91	+57
	162	250	250	231	220		
536	137	168	156	120	120	+31	−17

7. *The galactose tolerance test.*—Details of the tests on five rabbits given galactose intravenously are shown in Table IV. Duplicate estimations were made on separate samples of blood from four of the five rabbits.

In these experiments the criteria of liver damage were, first, a maximum concentration exceeding 160 mgm., second, a maximum rise exceeding 56 mgm., and third, a failure to return to the fasting concentration at the end of the test. A smaller dose of galactose would have been to advantage, and permitted the application of criteria precisely similar to those employed in the lævulose tolerance test. This, however, had little or no bearing on the significance of the results.

Negative results were obtained from four of the five rabbits on the first day of experiment. On the second day the tests on rabbits 531A, 533A and 522 were positive, while those on 535 and 536 showed little abnormality. Similarly, on the third day positive results were obtained from three rabbits, and clearly negative results from the other two. All were then killed and proved to have severe liver damage.

The five rabbits which had galactose intra-peritoneally yielded evidence of liver dysfunction as early as the first day of experiment. In these experiments the criterion of damage concerned the increase in blood-sugar content at one hour after administration of galactose. The averaged rise, when healthy, was by 66 mgm.; on the first day it was 113 mgm., on the second 260 mgm., on the third 157 mgm., and on the fourth day 199 mgm. There was, also, a failure to return to the fasting level three hours after dosage, but this discrepancy was notable only on the fourth day, when the blood-sugar content at the end of the test exceeded the fasting level by 96 mgm.

The experiments, therefore, show that the galactose tolerance test provided a better means of detecting liver damage than the lævulose tolerance test. When administered intravenously galactose yielded clearly positive results on the second day of experiment in three of five rabbits, whereas such results with lævulose were not obtained until the fourth day. This superiority was also shown in those experiments where the sugar was administered intraperitoneally. Liver damage was detected on the first day with galactose, but the test with lævulose was negative until the third day of experiment. One fault common to galactose and lævulose tolerance tests was their inability to detect liver damage on all occasions, as evidenced by the negative tests on rabbits 535 and 536 (galactose) and 529A (lævulose).

SUMMARY AND CONCLUSIONS.

The following modern tests of liver efficiency were applied to rabbits which had acute liver necrosis due to the administration of shale oil: the phenoltetrachlorphthalein test, the rose bengal test, the Van den Bergh reaction, the lævulose and galactose tolerance tests; the urea and amino-acid concentration of the blood was also determined. The development of an opalescence of the serum during experiment precluded examination of the rose bengal test and limited observation of the phenoltetrachlorphthalein test. The results obtained with the latter, prior to experiment, showed that it was unlikely to be a reliable test. Coincident renal dysfunction was responsible in some measure for increase in urea concentration of the blood, and prevented an estimation of the severity of liver dysfunction. A high concentration of amino-acid in the blood indicated gross liver damage, but was observed only as a

terminal phenomenon. The results of the lævulose tolerance test were equivocal, and it was not until the fourth day that unqualified positive results were obtained. A positive result was invariably associated with gross liver damage, but severe damage was present in a rabbit which gave a negative result on the third day. The galactose tolerance test was negative in two rabbits which had liver necrosis, but that lesion was invariably present when the test was positive. Liver damage was clearly indicated by this test, even on the second day of experiment in three of the five rabbits. Of the seven tests examined this one is most likely to give reliable indication of the presence of liver damage, and is probably able to detect damage of less severity than that which produced positive results in the other tests.

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THE PARALLEL ACTION OF NEUTRAL SALTS ON THE INHIBITION OF COMPLEMENT AND ON THE DISPERSION OF GELATIN.

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H. SACHS (1929, *a*) gives a brief summary of the inhibitory effects of salts on complement activity. Beyond suggesting that the inhibition is due, at least in part, to the formation of inactive complexes between the complement and the salts or their ions, Sachs makes no attempt to explain the results which he summarizes. Muir (1931) has also reviewed the subject of salt effects, but does not offer any general explanation of the very puzzling mass of results now available; whilst Hecht (1923) finds in salt-inhibition the only exception to his rule that inactivation is always accompanied by a lowering in surface tension. It is the view, however, of the present writers that the study of salt-inhibition has not been sufficiently systematic from the physico-chemical standpoint. Salts of widely differing types have often been used without due recognition of their differences, with the consequence that the results obtained have proved very complex and difficult to interpret. It is the purpose of this paper to show that the inhibitory action of certain salts, *i. e.* strictly neutral salts of sodium and potassium, runs parallel with their dispersive action upon gelatin, and to offer further support to the widely-held view that complement activity is closely bound up with the state of aggregation of the serum proteins (Browning, 1931; Sachs, 1929, *b*; Wells, 1929). No doubt an increase in aggregation can also cause inhibition, but it will be shown that the effect of the particular salts under consideration is, in all probability, to produce inhibition by increasing the dispersion. Such a view is directly suggested for sodium chloride by the well-known experiment of Muir and Browning (1909), which showed that complement inactivated by a hypertonic concentration of this salt would pass freely through a Berkefeld candle, whilst untreated active complement was largely retained by the filter.

The work of Wright and MacCallum (1922), referred to by Muir, is probably the most complete study of the inhibition of complement activity by salts that has yet appeared. These workers did not, however, discriminate sufficiently in their selection of salts. Their results are consequently in many cases due to the interaction of two or more effects, and do not, as a whole, lead directly to any theoretical insight into complement inhibition by salts.

In their Table V (p. 323) they give the following list of salts, arranged according to diminishing power of inhibition :

Na_3PO_4, Na citrate, $Ba(NO_3)_2$, $BaCl_2$, NaH_2PO_4, NaF, $CaCl_2$, $(NH_4)_2SO_4$, Na tartrate, Na_2HPO_4, Na_2SO_4, K_2SO_4, $NaHCO_3$, NaI, $NaNO_3$, KI, NH_4Cl, KNO_3, NaCl, KBr, KCl, Na acetate.

Of these twenty-two salts, no less than nine give acid or alkaline solutions in water, and are therefore unsuitably introduced into a study of simple salt effect. Trisodium phosphate gives a very alkaline solution ; sodium citrate, tartrate, acetate, bicarbonate and disodium phosphate give slightly alkaline solutions ; the ammonium salts, on the other hand, give slightly acid solutions, and monosodium phosphate gives a solution that is distinctly acid. Wright and MacCallum were fully aware of the lack of neutrality of many of the salts they used, but did not sufficiently recognize the ambiguity thereby introduced into their results. Further, salts with di- and trivalent ions were used, and compared with salts of monovalent ions on a basis of molarity and not of normality. The use of barium and calcium salts is not free from objection, since they give precipitates with proteins, under certain conditions, that are not completely reversible.

The anion series for complement inhibition given by Wright and MacCallum (vii, p. 322) is as follows :

$$PO_4'' > \text{Citrate} > F' > \text{Tartrate} > SO_4'' > I' > NO_3' > Cl' > \text{Acetate},$$

a series which invites comparison with that of Hofmeister for the precipitation of egg-albumen, though showing no immediately obvious resemblances. If those salts to which objection has been raised are omitted, Wright and MacCallum's series becomes—

$$SO_4'' > I' > NO_3' > Cl',$$

and if the moles of sulphate are recalculated to equivalents we get—

$$I' > NO_3' > SO_4''/2 > Cl'.$$

The lyotrope (Hofmeister) series for the swelling of neutral gelatin is, omitting certain salts, usually given as—

$$CNS' > I' > Br' = NO_3' > Cl' > (H_2O) > SO_4''.$$

It will be noted that with the exception of sulphate the ions of Wright and MacCallum's series are in the lyotrope order for protein swelling ; they are also in correct order for the solubilization of gelatin, and for the lowering of both the setting temperature and the modulus of elasticity for gelatin jellies.

Such considerations as have been so far adduced led the writers to believe that a reinvestigation of the inhibitory effects of certain salts would be valuable, and a study has therefore been made of the following two series of salts :

Na_2SO_4, NaCl, NaBr, $NaNO_3$, NaI, and NaCNS

and—

K_2SO_4, KCl, KBr, KNO_3, and KI.

With the exception of sodium thiocyanate, these are all salts of both strong acids and strong bases, and give neutral solutions. For this reason, and because they cannot act as buffers, they will not affect the pH of serum. Sodium thiocyanate is not a strictly neutral salt, but its solutions are so nearly at the pH of serum (pH = 7·4 for N/2 NaCNS) that there can be no objection to their feeble alkalinity.

Twice-normal solutions were prepared of all the salts except the sulphates, of which normal solutions were made, and the thiocyanate, of which a 1·20N solution was used.

The mode of experiment adopted was as follows: Quantities of 0·1, 0·2, up to 0·5 c.c. of salt solution were added to 2 c.c. (in some cases 1·0 or 0·5 c.c.) quantities of guinea-pig serum. The mixtures were then allowed to stand at room temperature for two hours, after which quantities of 0·05, 0·10, or 0·20 c.c. of the mixtures were added to 0·10 c.c. of a 10 per cent. suspension of sensitized ox-cells in 0·85 per cent. sodium chloride. After incubation at 37° C. for two hours the degree of hæmolysis was read and recorded under five headings, namely, complete hæmolysis, almost complete, partial, trace of hæmolysis, no hæmolysis.

The inactivating titre was taken as the mean of the smallest amount of salt which caused complete inhibition, and that amount which allowed a trace of hæmolysis to persist. This was considered to be the most reliable criterion for estimating the inhibitory concentration of the salts used. It may here be pointed out that Wright and MacCallum did not determine the *inactivating* titre, but the smallest amount of salt which prevented *complete* hæmolysis. Further, these workers arranged their experiments so as to have a constant (isotonic) concentration of sodium chloride in all their tests. This has not been done in the work here described, since it was considered that small variations in the sodium chloride would be of little moment, and also because the sodium chloride is strictly constant for any horizontal row in the tables of results (except, of course, where sodium chloride is the added salt under test). The 10 per cent. suspension of ox-cells was used in small volume (0·10 c.c.) so as to avoid unnecessary dilution of the salt-complement mixture. This is not unimportant in view of the reversibility of salt-inhibition. It will be seen from the tables that experiments strictly parallel except for the amount of salt-complement mixture used, show as a rule greater persistence of hæmolysis with the smaller volume of mixture, *i. e.* with greater dilution by the fixed volume of blood suspension, and consequently greater reversal of the salt-inhibition by dilution. Neat serum was generally used so as to avoid possible complications due to alteration of the ratio of complement to sodium chloride in the serum by diluting with isotonic salt solution. However, one or two experiments with 50 per cent. and 25 per cent. serum did not give any different results, except that inhibition was generally effected at lower salt concentrations.

The following tables give actual results obtained. Each table is the complete record of an experiment, but only a small proportion of the total data obtained are here reproduced. Many repetitions of the experiments were carried out, with results invariably in agreement with those given in the tables.

TABLE I.—*Potassium Salts.*

c.c. salt solution added to 2 c.c. serum.	Normality of salt in serum.	Degree of hæmolysis.							
		KCl.		KNO_3.		KBr.		KI.	
		c.c. mixture.		c.c. mixture.		c.c. mixture.		c.c. mixture.	
		0·10.	0·20.	0·10.	0·20.	0·10.	0·20.	0·10.	0·20.
0·10	0·095	++++	++++	++++	++++	++++	++++	++++	++++
0·20	0·180	++++	++++	++++	++++	++++	++++	++++	++++
0·30	0·260	++++	++++	++++	++++	++++	+++	+	..
0·40	0·330	++++	++++	++++	+	+++	+	..	..
0·50	0·400	++++	+++	+	..	..	..	..	..

0·1, 0·2, etc., c.c. of salt solution added to 2 c.c. serum. Allowed to stand 2 hours, then 0·1 and 0·2 c.c. of mixture added to 0·1 c.c. of 10 per cent. suspension of sensitized ox-cells and incubated at 37° C. for 2 hours. Degree of hæmolysis then read.

++++ = complete hæmolysis, +++ = nearly complete, ++ = partial, + = trace of hæmolysis, and .. indicates no hæmolysis, *i. e.* complete inhibition.

TABLE IVA.—*Sulphates and Chlorides.*

NaCl.		Na_2SO_4.	KCl.		K_2SO_4.	
Normality of salt in serum.	Hæmolysis. 0·10 c.c. mixture added to 0·05 c.c. blood.	Normality of salt in serum.	Normality of salt in serum.	Hæmolysis. 0·10 c.c. mixture added to 0·05 c.c. blood.		Normality of salt in serum.
0·095	++++	++++	0·09	0·095	++++	++++	0·09
0·180	++++	++++	0·17	0·180	++++	++++	0·17
0·260	++	++	0·23	0·260	+	++	0·23
0·330	..	..	0·29	0·330	+	..	0·29
0·400	..	..	0·33	0·400	..	..	0·33

0·1, 0·2, etc., c.c. of chloride solutions (2N.) added to 2 c.c. serum ; 0·1, 0·2, etc., c.c. of sulphate solutions (N.) added to 1 c.c. serum. After standing, 0·1 c.c. of mixture added to 0·05 c.c. of 10 per cent. suspension of ox-cells and incubated.

TABLE IVB.

NaCl.		Na_2SO_4.	KCl.		K_2SO_4.	
Normality of salt in serum.	Hæmolysis. 0·10 c.c. mixture added to 0·05 c.c. blood.	Normality of salt in serum.	Normality of salt in serum.	Hæmolysis. 0·10 c.c. mixture added to 0·05 c.c. blood.		Normality of salt in serum.
0·18	++++	++++	0·17	0·18	++++	++++	0·17
0·33	+	+	0·29	0·33	+	+	0·29
0·46	..	..	0·38	0·46	..	..	0·38

0·1, 0·2, etc., c.c. of chloride solutions (2N.) added to 1 c.c. serum ; 0·1, 0·2, etc., c.c. of sulphate solutions (N.) added to 0·5 c.c. serum. After standing, 0·1 c.c. of mixture added to 0·05 c.c. of 10 per cent. suspension of ox-cells, and incubated.

TABLE IIA.—*Potassium Salts.*

c.c. salt solution added to 2 c.c. serum.	Normality of salt in serum.	Hæmolysis. K_2SO_4. c.c. mixture.		c.c. salt solution added to 2 c.c. serum.	Normality of salt in serum.	Degree of hæmolysis. KCl. c.c. mixture.		KNO_3. c.c. mixture.		KBr. c.c. mixture.		KI. c.c. mixture.	
		0·10	0·20.			0·10.	0·20.	0·10.	0·20.	0·10.	0·20.	0·10.	0·20.
0·10	0·05	++++	++++	0·10	0·095	++++	++++	++++	++++	++++	++++	++++	++++
0·20	0·09	++++	++++	0·20	0·180	++++	++++	++++	++++	++++	++++	++++	++++
0·30	0·13	++++	++++	0·30	0·260	++++	++++	++++	++++	++++	++++	+	..
0·40	0·16	++++	++++	0·40	0·330	++++	++++	+++	++	+++	++	..	..
0·50	0·20	++++	++++	0·50	0·400	++++	+++	+	..	+	..	..	..

Experiment conducted exactly as for Table I.

TABLE IIB.

c.c. salt solution added to 2 c.c. serum.	Normality of salt in serum.	Degree of hæmolysis. KCl. c.c. mixture.		KNO_3. c.c. mixture.		KBr. c.c. mixture.		KI. c.c. mixture.	
		0·10.	0·20.	0·10.	0·20.	0·10.	0·20.	0·10.	0·20.
0·10	0·095	++++	++++	++++	++++	++++	++++	++++	++++
0·20	0·180	++++	++++	++++	++++	++++	++++	..	..
0·30	0·260	++++	++++	++	..	++	+	..	..
0·40	0·330	+++	+	..	..	..	..	..	..
0·50	0·400	+	..	..	..	..	..	..	..

As Table IIA, but using 50 per cent. serum and omitting sulphate.

TABLE IIC.

c.c. salt solution added to 2 c.c. serum.	Normality of salt in serum.	Degree of hæmolysis. KCl. c.c. mixture.		KNO_3. c.c. mixture.		KBr. c.c. mixture.		KI. c.c. mixture.	
		0·10.	0·20.	0·10.	0·20.	0·10.	0·20.	0·10.	0·20.
0·10	0·095	++++	++++	+++	++	++++	++++	++	+
0·20	0·180	++++	++++	+++	++	+++	+++	..	..
0·30	0·260	+++	++	..	..	..	..	..	..
0·40	0·330	Not done		..	..	..	..	..	..
0·50	0·400	Do.		..	..	..	..	..	..

As IIA and IIB above, but using 25 per cent. serum.

TABLE III.—*Sodium Salts.*

c.c. salt solution added to 2 c.c. serum.	Normality of salt in serum.	Degree of hæmolysis.										Normality of NaCNS in serum.
		NaCl.		$NaNO_3$.		NaBr.		NaI.		NaCNS.		
		c.c. mixture.		c.c. mixture.		c.c. mixture.		c.c. mixture.		c.c. mixture.		
		0·05.	0·10.	0·05.	0·10.	0·05.	0·10.	0·05.	0.10.	0·05.	0·10.	
0·10	0·095	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	0·11
0·20	0·180	++++	++++	++++	++++	++++	++++	++++	++	++	++	0·20
0·30	0·260	++++	++++	++++	++++	++++	++++	+++	..	+	..	0·24
0·40	0·330	++++	+	++	+	+++	+	+	..	—	—	—
0·50	0·400	++++	..	+	..	+	..	..	..	—	—	—

Experimental details as for Table I, except that 0·05 and 0·1 c.c. of salt-complement mixture used instead of 0·1 and 0·2 c.c.

It is clear from the tables given that the anion series in order of diminishing inhibitory power is :

$$CNS' > I' > Br' = NO_3' > SO_4''/2 \geqslant Cl'.$$

The accuracy of the experiments hardly allows us to say definitely whether $SO_4''/2$ is equal in effect to Cl′ or a little stronger. Inactivating titres for 100 per cent. serum, calculated for experiments in which practically complete inhibition was obtained with most of the salts, are given in Table V, the results being expressed as normality of added salt in the serum :

TABLE V.—*Inactivating Titre of Anions.*

CNS′.	I′.	Br′.	NO_3'.	Cl.	$SO_4''/2$.
..	0·30	0·37	0·40	0·40	0·45
..	0·26	0·30	0·30	0·40	0·38
..	0·17	0·25	0·25	0·35	..
..	0·23	0·35	0·35	0·43	0·32
0·18	0·26	0·33	0·33	0·46	..
0·20	0·33	0·33	0·33	0·46	0·44

N.B.—Complete inhibition by sulphates was not obtained with some specimens of serum owing to the lower concentration (N/1) of the sodium and potassium sulphate solutions used.

These figures are typical of 31 similar series. Of course, perfect reproducibility cannot be expected in experiments of this nature, and there is occasionally a little variation in results. Within the limits of accuracy, bromide was usually found to be equal to nitrate, but sometimes appeared to be a little stronger, sometimes a little weaker. In two experiments bromide was found to be equal to chloride. This must have been due to error in technique. The inactivating titre for each salt varied considerably with different specimens of serum, as can be seen from the vertical columns ; the titre for chloride, for example, varied from 0·33 N. to 0·57 N., and for iodide from 0·18 N. to 0·33 N. No reason for this is advanced except that it may be due to the " amount " of complement present in the serum, since experiments showed (see Table II) that the activity of a diluted serum was inhibited by smaller concentrations of salts than were required for the neat serum.

DISCUSSION.

The anion series for complement inhibition,

$$CNS' > I' > Br' = NO_3 > SO_4''/2 > Cl',$$

is, except in one particular, the same as the lyotrope series for the swelling of gelatin as usually quoted. The exception is the position of sulphate. It should be pointed out, however, that the lyotrope series is not always found in exactly the same form in all cases for which it has been studied. For example, the differences in ion effects shown by the swelling action of sodium salts on gelatin do not remain quite the same at all concentrations of the salts.

It is important, therefore, to consider the action of salts in the concentration region of 0·2–0·5 N., *i.e.* roughly the range of concentrations used in the experiments on hæmolysis.

Much light is thrown on this question by the recent work of Stiasny, Das Gupta and Tresser (1925), and of Northrop and Kunitz (1925). Stiasny studied in the first place the swelling of gelatin in solutions of sodium thiocyanate, nitrate, chlorate, chloride and sulphate. Omitting chlorate, which has not been used in the hæmolysis experiments, Stiasny obtains the following series :

At 0·10 N. conc. $CNS' > NO_3' > SO_4''/2 > Cl'$ (small difference between SO_4'' and Cl').

„ 0·20 N. „ $CNS' > NO_3' > SO_4''/2 = Cl'$.

„ 0·50 N. „ $CNS' > NO_3' > Cl' > SO_4''/2$.

„ 1·00 N. „ $CNS' > NO_3' > Cl' > SO_4''/2$.

Stiasny's experiments were carried out with carefully prepared iso-electric and ash-free gelatin. The solutions of salts were carefully examined before and after the swelling experiments in order to ensure that no pH changes had occurred which might affect the results.

At *low* concentrations there is little difference in swelling action between sodium chloride and sodium sulphate—behaviour very different from that of the same two salts at higher concentrations. In the stronger solutions of thiocyanate and nitrate and in very concentrated (4 N.) solutions of chloride the gelatin passed into solution, but without being hydrolyzed, as was shown by formaldehyde titrations, and van Slyke amino-nitrogen determinations, carried out on the dissolved gelatin. No increase in carboxyl or amino groups could be detected as compared with the original material. Stiasny consequently regards the swelling action of thiocyanate, nitrate and chloride as a "peptic" dispersion effected without rupture of any ordinary chemical linkages. The action of sulphate may be regarded as dispersive at low concentrations but coagulative in stronger solutions. In order to obtain further evidence for this point of view Stiasny has also studied the action of salts on other properties of gelatin. The speed of ultra-filtration of gelatin solutions was found to be much reduced by the addition of sodium thiocyanate, and more gelatin passed through the filter. Gelatin with added thiocyanate passed through a dialysing membrane which was impermeable to untreated gelatin. Stiasny has also made the striking observation that neutral salts diminish the optical activity of gelatin in the same way as gentle heating. The order of effectiveness of salts at N/1 concentration is :

$$CNS' = I' > NO_3' > Cl' > (H_2O) > SO_4''.$$

On the basis of the above experiments, and of others such as tannin precipitation and protective colloid action, Stiasny concluded that the action of neutral salts is, except in the case of sulphate at higher concentrations, to increase the dispersion of gelatin without producing chemical alteration.

This view is confirmed by the work of Northrop on the swelling of solid gelatin, and on the osmotic pressure of gelatin solutions in the presence of salts. Northrop found that 0·5 N. solutions of sodium iodide, bromide, nitrate and chloride at 0° C. increase the swelling of gelatin in the order—

$$I' > Br' = NO_3' > Cl'.$$

At higher concentrations the order is—

$$I' > Br' > NO_3' > Cl'.$$

In his osmotic pressure experiments at 37° C. Northrop finds that low concentrations of these salts all increase the osmotic pressure, but that at these low concentrations the differences between the effects of the different salts are so very small that the actual anion order is not very clear, though it appears to be—

$$I' = NO_3' > Br' > Cl'.$$

At higher concentrations the order

$$I' > Br' > NO_3' > Cl'$$

is clearly shown.

Northrop considers that the salts influence the osmotic pressure of the gelatin itself, and that the effects on osmotic pressure and swelling are closely parallel. Since an increase in osmotic pressure can only result from an increased dispersity of the gelatin, the conclusion to be drawn is that the effect of nitrates, iodides, bromides and chlorides in low concentration is to increase the dispersity of the gelatin. Northrop implies agreement with Stiasny's conclusions, and confirms the latter's statement that the action of neutral salts does not cause hydrolysis.

To sum up, the series for the effect of anions in increasing the dispersity of gelatin is, according to the most recent work—

$$CNS' > I' > Br' = NO_3' > SO_4''/2 \geqslant Cl'$$

at low concentrations of the sodium salts. This series is exactly that obtained for the inhibition of complement activity by neutral salts. In the opinion of the present writers, this constitutes very strong evidence in favour of the view that the inhibitory action of the neutral sodium and potassium salts described in this paper is due to a dispersion or peptization of the protein complexes of the serum. Admittedly gelatin is not one of the serum proteins, but it is very unlikely that the effect of neutral salts on the osmotic pressures of serum proteins would give results in any way significantly different from those obtained with gelatin.

It is, of course, not maintained that the inhibitory effect of salts of the alkaline earths or of heavy metals is due to the dispersion of serum proteins. Many salts no doubt cause coagulation or aggregation of the proteins—an effect which is just as much a disturbance (though in the opposite sense) of the original physico-chemical state of the serum proteins as is the dispersion discussed in this paper, and therefore equally capable of causing inhibition. The dispersion hypothesis, however, offers a very natural explanation of the experiment of Muir and Browning already referred to, and also explains why neutral salt action is exceptional according to Hecht. Dispersion of a protein will increase surface tension rather than diminish it.

In conclusion it may be stated that Stiasny has found that the dispersion by thiocyanate is largely, but not entirely reversible. Data on other salts do not appear to be available at present, and consequently no general statement on reversibility can be made. It is interesting, however, that neutral salt inhibition of complement is usually considered to be largely if not completely reversible.

SUMMARY.

A study has been made of the action of neutral, non-hydrolysable salts of sodium and potassium on the inhibition of complement. The results give the following anion series, in order of diminishing effectiveness :

$$CNS' > I' > Br' = NO_3' > SO_4''/2 \geq Cl'.$$

This is the lyotrope series for the salt effects in the swelling of purified gelatin at *low* concentrations of salts. The work of Stiasny, Das Gupta and Tresser, and of Northrop and Kunitz, shows that the action of neutral salts at low concentrations is to produce dispersion of gelatin without chemical decomposition, as is shown not only by the effects on swelling, but on osmotic pressure and other properties. It is argued that under the conditions of the experiments described in this paper, a dispersion of the serum proteins is the most probable cause of the inhibition observed, and that complement activity is necessarily associated with a particular state of aggregation of the serum proteins.

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OPTIMAL PROPORTIONS IN AGGLUTINATION: WITH REFERENCE TO THE ANTIGENIC ANALYSIS OF THE *BRUCELLA* GROUP OF ORGANISMS.

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In 1926 Dean and Webb described a method of titration of varying amounts of antigen against a constant volume of precipitating antiserum, in which the proportion of the two reagents in the most rapidly flocculating mixture was determined. This " optimal " proportion was independent of the absolute concentrations of antigen and antiserum. Subsequent work demonstrated the value of the method as a means of titrating the antigen or precipitating antibody concentration in a solution, the concentration of the other reagent being known (Dean and Webb, 1928 ; Taylor, 1931 ; Wells, 1931 : using serum proteins and specific antisera. Taylor, Adair and Adair, 1932 : crystalline egg albumen and specific antisera. Wilson Smith, 1932 : titration of anti-pneumococcal sera with polysaccharide).

The Ramon test is an example of the reverse method of titration ; here the diphtheria toxin is kept constant, and the concentration of antitoxin varied. The mixture which particulates most rapidly is taken as equivalent.

Proportions of bacterial suspensions and immune sera which are optimal for most rapid agglutination have been described by Heuer (1922), Costa Cruz (1929) and Bier (1931), who titrated a constant amount of antigen against varying serum concentrations.

The object of this communication is to show that optimal mixtures of immune sera and bacterial suspension are constant for given bacteria and antisera, and that their determination affords a basis for comparison of related bacterial species. The *Brucella* group was investigated, in the hope of finding a rapid direct method of serological typing. Previous work (Wilson and Miles, 1932) showed that reliable differentiation could be obtained by cross-absorption tests, or by the preparation and use of " mono-specific " sera, but not by direct agglutination.

EXPERIMENTAL.

Materials :

Four representative strains were used.

1. K 25 : Bovine abortus of human origin from Denmark.
2. Little : „ „ „ „ „ „ England.
3. P 1 : *Porcine* „ „ porcine „ „ New York State, U.S.A.
4. Y 6 : *Melitensis* „ human „ „ Palestine.

Salt- and heat-agglutination tests for roughness were negative (Pandit and Wilson, 1932). Strains K 25, P 1 and Y 6 were used in the preparation of antisera. Later, partially rough variant colonies appeared in K 25, and the bovine abortus Little was used in its place as a smooth, fully homologous agglutinating suspension.

Suspensions were made by washing off three-day heart broth agar cultures with 0·5 per cent. formol saline, heating the washings for one hour at 56° C., spinning down and resuspending the sediment in 0·25 per cent. formol saline. Antisera, prepared by immunizing rabbits, were preserved with 0·3 per cent. phenol.

Technique of the Agglutination and Precipitin Tests.

Monax glass tubes of uniform bore (0·8 × 120 cm.) were used, giving a column of fluid 4·5 cm. high when filled with 1 c.c. of each reagent. The fluid columns were half-immersed in a 52° C. illuminated water-bath, and read directly through a glass window against a black background. Standard particulation was arbitrarily fixed as that degree visible at a glance through a reading-glass 3½ in. in diameter and of 9½ in. focal length. This degree can be timed in a single tube to within ± 15 seconds if the particulation time is not more than 40 minutes. With longer times, accuracy is diminished. To obviate the errors arising from imperfect mixing (Wilson Smith, 1932), the reagent added second was squirted in forcibly from a 1 c.c. tuberculin syringe, No. 1 needle, and the tubes timed from each squirting.

Preliminary rough tests with serial two-fold dilutions of the reagent to be varied were followed by fine tests, using a smaller dilution factor.

Serum and suspension concentrations are recorded as those of the unmixed reagents, not as the final concentration of each in the mixtures. The concentration of bacterial suspensions in optimal tubes was first measured by direct counts in a Helber chamber, but when it was evident that the differences in optimal concentrations were large enough to be measured by opacity methods, all suspensions were standardized against a comparison tube containing a counted 2,000 million *Br. abortus* per c.c., and made up in stock solutions of 10,000 million per c.c. opacity.

The Constant-Antiserum Titration.

Table I shows a typical fine titration, and Table II the results with all three antigens and their antisera. The figures for each antiserum show that

melitensis is differentiable from the more closely related *abortus* strains, and that in each series the more markedly heterologous strain has a lower optimum concentration. The bovine and porcine *abortus* strains do not differ significantly.

A suitable serum dilution giving an optimum with a workable concentration of bacteria has first to be found. Reading is difficult above a final concentration of $5{,}000 \times 10^6$ per c.c., and below one of 300×10^6 per c.c. The chief difficulty of the method is the comparison of particulation rates in a series of tubes of differing initial opacities.

TABLE I.—*Constant-Antiserum Titration of Porcine Abortus Serum P 1 (1/400) Against P 1, Y 6, and Little.*

Antigen, in 10^9 per c.c.	Suspension.			Optimal concentration, 10^9 per c.c.
	P 1.	Y 6.	Little.	
9·5	..	..	..	..
9·0	>22	..	..	..
8·5	21	..	26	P 1, 8·2
8·0	21	..	24	Little, 8·0
7·5	>22	..	26	..
7·0	..	..	..	..
6·5	..	..	..	..
6·0	..	..	..	..
5·5	..	..	..	..
5·0	..	>46	..	..
4·5	..	45	..	..
4·0	..	45	..	Y 6, 4·0
3·5	..	45	..	..
3·0	..	>46	..	..

Particulation time in minutes. Bath 52° C.

TABLE II.—*Results of Constant-Antiserum Tests.*

Sera.	Optimal concentration of antigens in 10^9 per c.c.		
	Little.	P 1.	Y 6.
K 25 (1/800)	7·0	7·0	5·0
P 1 (1/400)	8·0	8·2	4·0
Y 6 (1/300)	2·0	2·1	2·9

The Constancy of the Constant-Antiserum Optimal Ratio.

To determine whether the optimal ratio was constant and independent of the concentration of the reacting system, a series of constant-antiserum titrations were made, doubling the dilution of antiserum in each successive test. The results of one such test are shown in Table III.

An optimum concentration of $8{\cdot}0 \times 10^9$ per c.c. appears in the eighth test, at serum-dilution 1/160. A two-fold dilution of the serum, and the optimum shifts to $4{\cdot}0 \times 10^9$ per c.c., and so to $2{\cdot}0 \times 10^9$ per c.c. for serum at 1/640. The ratio remains the same throughout. Finer tests, with smaller serum dilution-factors, bear this out for antigen concentrations between $10{\cdot}0 \times 10^9$ and $1{\cdot}0 \times 10^9$ per c.c.

TABLE III.—*Particulation Velocities in Minutes in Constant-Antiserum Titration of Serum P1 and its Antigen.*

Serum dilution used in each test.	Antigen concentration in 10^9 per c.c.				
	16·0.	8·0.	4·0.	2·0.	1·0.
1/1	2½	4	6½	15	32
1/2	2	4	7	11½	25
1/5	3½	4	8	16	34
1/10	3½	4¾	8½	15½	48½
1/20	4	6	9	15	40
1/40	5	7	13	23½	49
1/80	6	6	11	23	73
1/160	10	9	12	20	51
1/320	21	15	14	28	43
1/640	>90	49	30	27	47
1/1280	>100	>100	99	60	55

The Constant-Antigen Titration.

The columns in Table III may be considered as the result of constant-antigen titrations against varying antiserum. If the velocities of particulation are plotted against the logarithm of the serum dilution, as in Fig. 1, a series of constant-antigen curves is produced. For antigen $16{\cdot}0 \times 10^9$, the velocity increases smoothly with increasing concentration of antiserum, a finding noted by Taylor (1931) using horse-serum and its antiserum, and Eagle (1932) in both precipitin and agglutination tests. But, as Taylor states in a later paper (1933), increase in antiserum concentration may lead to slowing of the particulation rate if the antigen is dilute enough.

The curves in Fig. 1 show slowing of the particulation rate in high serum concentrations at an antigen concentration of $2{\cdot}0 \times 10^9$, two optima appearing

at serum dilutions approximately 1/2 and 1/200. A further twofold antigen dilution reveals three definite optima.

The relation of the constant-antiserum optimum to the constant-antigen curves is clearly shown in Fig. 1.

Taking the curve A for an antigen concentration of $16{\cdot}0 \times 10^9$ per c.c., as serum concentration decreases, the particulation time increases, until at a serum 1/500 the curve turns almost parallel to the base-line. The particulation time becomes indefinitely large if the serum concentration is further decreased, and for 1/1000 is greater than 48 hours; 1/500 represents the end-point (24-hour reading) of the ordinary agglutination titration for that antigen concen-

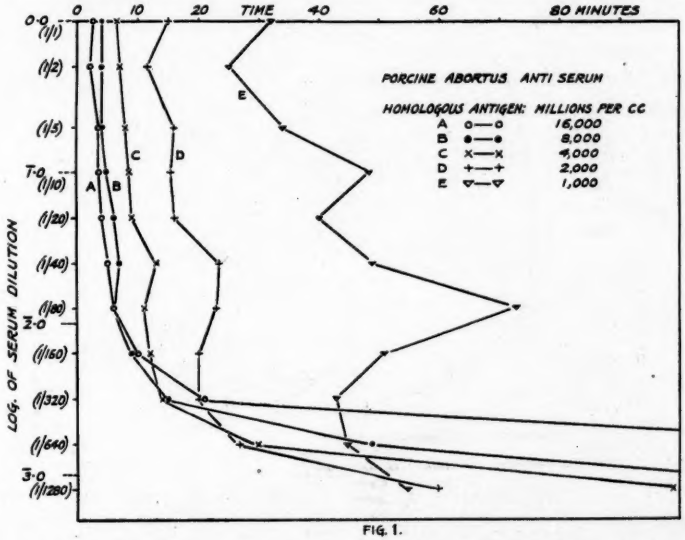

FIG. 1.—Particulation velocity curves for constant doses of antigen. (See Table III.)

tration. The curve B, for antigen half as concentrated, lies to the right of A in high serum concentration, and crosses A at the 1/80 level. That is, suspensions of $16{\cdot}0 \times 10^9$ and $8{\cdot}0 \times 10^9$ per c.c. particulate equally fast with serum 1/80, and the constant-antiserum optimum lies between these two antigen values. In still lower serum concentration B lies to the left of A; antigen $8{\cdot}0 \times 10^9$ per c.c. will always particulate faster than antigen $16{\cdot}0 \times 10^9$ per c.c. at concentrations less than 1/80. Similarly C crosses A and B, and immediately after crossing B at about 1/300 represents the fastest system of those plotted, until at about 1/600 it is crossed in turn by D, which becomes the optimal system at 1/640.

These constant-antigen curves and those in Fig. 2, which are plotted from a

series of constant-antigen titrations, show (*a*) that dilution of the antigen unmasks optima, (*b*) that the more dilute the antigen the more clearly defined the optimum, and (*c*) that the lower optima are produced by a proportion of the reagents roughly constant for all the curves, and that these points occur at a serum concentration about 4 times as strong as the constant-serum optima (the serum level at which the curve comes to lie to the left of the others), which in turn occur in a serum dilution about 4 times as strong as the end titre of the serum.

The constant-antigen optimal ratio was constant for a given strain and its

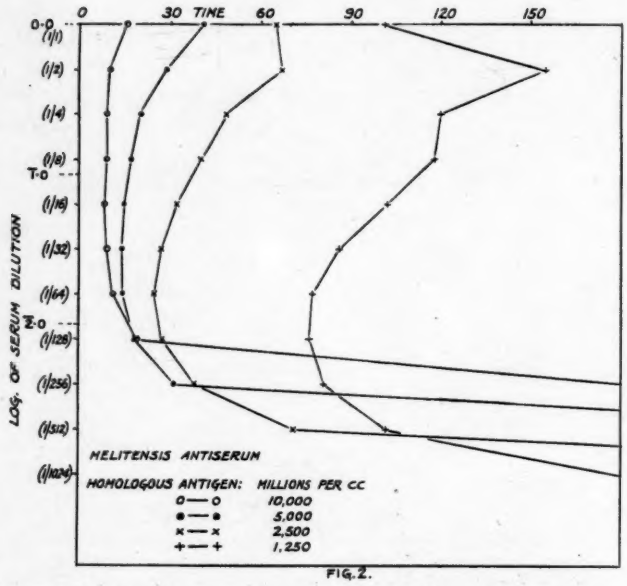

FIG. 2.—Particulation velocity curves. Constant-antigen titrations.

immune serum when fine titrations were made—a result confirming that of Costa Cruz (1929), who used *Bact. flexneri* and its specific antiserum.

The lower optima (*i. e.* those occurring in relatively high serum dilutions) for the three *Brucella* suspensions are shown in Table IV. Suspensions of $2{\cdot}5 \times 10^9$ per c.c. were used throughout; a lower concentration would have given sharper, though less rapidly read results. The average flocculation time for the optimal tube was about 25 minutes.

As with the constant-antiserum method, this titration differentiates the *melitensis* Y 6 from the two *abortus* strains with each serum. The optimal concentration is highest for the most heterologous strain.

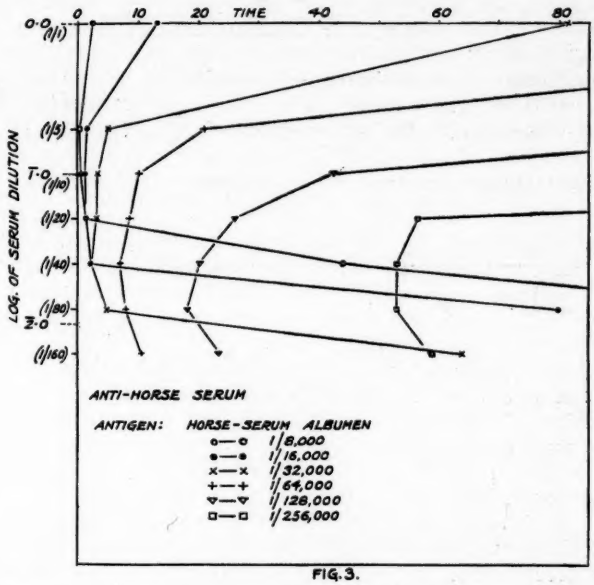

FIG. 3.—Particulation velocity curves in precipitation. Constant-antiserum titrations.

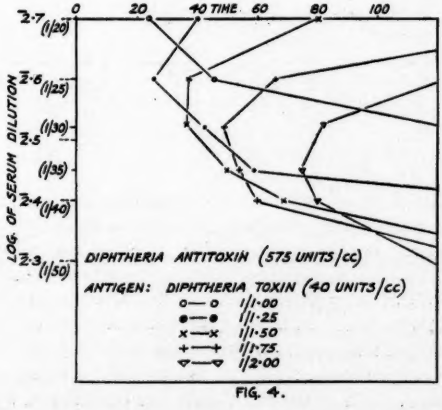

FIG. 4.—Particulation velocity curves in toxin-antitoxin reactions. Constant-antiserum titration.

TABLE IV.—*Results of Constant-Antigen Tests.*

Antigen 2·5 × 10^9 per c.c.	Optimal dilutions of antisera. K 25.	P 1.	Y 6.
Little	1/150	1/36	1/29
P 1	1/150	1/32	1/33
Y 6	1/80	1/18	1/80

The Significance of the "Upper" Optima.

At least one optimum occurred in all the *Brucella* sera, and in all of a limited number of anti-typhoid, anti-Aertrycke and anti-Shiga sera tested against homologous bacterial suspensions. Two optima were found only in high titre *Brucella* sera. No attempt has yet been made to titrate the upper optima against different species; neither has its nature been elucidated. Widely separated double zones occur in constant antiserum titrations of horse-serum and anti-horse-serum rabbit serum (Goldsworthy, 1928), and are probably the separate optima of two distinct antigens and their antibodies. Double zones of this kind were produced artificially by Taylor (1931), who titrated mixtures of anti-horse-sera possessing widely different ratios and flocculating times against horse-serum by the constant-antiserum technique. But the same author with Adair and Adair (1932) found double zones appearing in precipitin tests in which the antigen was apparently chemically unique.

Double precipitation zones in constant-antigen titrations did not occur when unique antigens like horse-serum albumen and diphtheria toxin were tested against their antisera (Figs. 3 and 4), and on this basis it is attractive to assume that another antigen is concerned in the production of the upper optima in *Brucella* titrations. Work is in progress to test the validity of this assumption; in this communication only the lower optima and their corresponding constant-antiserum optima will be considered.

(E. G. D. Murray, in a private communication to Goldsworthy (1928), reports double zones, at about 1/20 and 1/200, in an ordinary titration of anti-meningococcal horse-serum with meningococcal suspension. Priestley (1931) investigated a zone of inhibition occurring in agglutination tests of cow sera against *Br. abortus* at a dilution of about 1/80 to 1/160, and suggested that the zone of agglutination in the lower serum dilution was due to the reaction of an antigen and antibody different from those concerned in the zone of agglutination in the higher dilutions.)

The Nature of Optimal Proportions in Brucella *Agglutination.*

Dean and Webb, investigating the precipitin reaction between horse-serum and anti-horse-serum, could find no significant amount either of free antigen or of free antibody in the supernatant fluid from a constant-antiserum optimal mixture after precipitation, and concluded that the optimal mixture was an equivalent mixture. Their observations are confirmed by Marrack and Smith (1931) and by Wilson Smith (1932).

Using the precipitin reaction, two experiments with horse-serum and anti-horse rabbit serum showed that while the supernatant fluid in a constant-antiserum optimal mixture contained no antibody, the supernatant in a constant-antigen optimal mixture, using the same absolute quantity of antiserum, contained at least 50 per cent. of the antibody originally used, titrated by the constant-antiserum method. The full amount of residual antibody in the constant-antigen optimal mixture was not determined; the titration series used was not sufficiently extensive. The results with agglutination were different. Only antibody can be tested for in the supernatant fluid.

Equivalence in Constant-Antiserum Optimal Mixtures.

Experiment 1.

K 25 (bovine *abortus*) serum 1/800, put up against Little suspensions, $10{\cdot}0 \times 10^9$, $9{\cdot}0 \times 10^9$, $8{\cdot}0 \times 10^9$, etc., to $3{\cdot}0 \times 10^9$ per c.c. Optimum at $7{\cdot}0 \times 10^9$ in 20 minutes at 52° C. Left for 2 hours at 52° C., then all suspensions spun down and supernatants tested with a 500×10^6 per c.c. suspension of Little.

Result: Antigen concentrations from $10{\cdot}0$ to $6{\cdot}0 \times 10^9$ per c.c. removed all agglutinins; $5{\cdot}0$ to $3{\cdot}0 \times 10^9$, per c.c. left enough to give a "query trace" of agglutination after 20 hours at 56° C.

The constant-antiserum optimum is equivalent, but ratios widely spread on either side of this value are similarly equivalent.

Equivalence in a Constant-antigen Optimal Mixture.

Experiment 2.

K 25 serum, two-fold serial dilution, against Little suspension $2{\cdot}5 \times 10^9$ per c.c. Held at 52° C. Two optima appeared, at 1/16 and 1/256, in 29 and 24 minutes respectively. After 75 minutes, when all the tubes showed marked agglutination the bacteria were spun down, and the supernatants titrated, with five-fold dilutions, against a 500×10^6 per c.c. suspension of Little. Table V shows the result. The lower constant-antigen optimum is equivalent. The equivalence is spread on either side of the ratio and includes the constant-antiserum ratio, which for this antigen concentration is at a serum dilution of 1/2000.

It is noteworthy that the upper optimum is not equivalent.

The exact point of equivalence is difficult to determine. A shorter absorption period, one even less than the time required to produce standard particulation in the optimal mixture, gives the same widespread zone of total absorption.

The results confirm the findings of Heuer (1922), who found the same widespread zone of equivalence round the constant-antigen optima of typhoid and Flexner-immune sera. Costa Crux (1929) finds residual agglutinins in the supernatant fluid from such an optimal mixture, but in smaller amount than in any of the other tubes in the titration.

TABLE V.—*Absorption Test, Constant-Antigen Titration K 25 Serum Against Little.*

Serum dilution.	Titre of supernatant after agglutination with $2{\cdot}9 \times 10^9$ per c.c. suspension.
1/2	Trace 3125
1/4	,, 625
1/8	Total 125
1/16 (upper optimum)	Trace minus 125
1/32	,, 25
1/64	Trace 1
1/128	<1
1/256 (lower optimum)	<1
1/512	<1
1/1024	<1
1/2048	<1

DISCUSSION.

There are, then, three titrations available for testing a bacterial suspension against an antiserum: a constant-antigen end-point, a constant-antigen optimum, and a constant-antiserum optimum. The first two, occurring in the same sytem, are related as follows: The 24-hour end-point represents the smallest amount of serum which can sensitize a given dose of antigen to produce particulation in that time. Increase in the serum concentration leads to more rapid particulation, until a point is reached when the antigenic "groups" on the bacterial surface are all associated with antibody. Increase in excess of this equivalent point leads to slowing of the reaction, though the bacterial surface is still capable of binding antibody a little in excess of equivalence. The explanation of the slowing by excess antibody is doubtful, but some preliminary experiments suggest that the "agglutinoid" theory recently revived in a modified form by Shibley (1929) will not fit the facts.

The optimum occurs in serum about 16 times as concentrated as the end-titre. In this connection the findings of de Kruif and Northrop (1922) are pertinent. These workers defined as unit agglutinin the amount of antiserum which gave an end-titre with a constant dose of typhoid bacilli. They found that this dose of bacilli could bind up to 16 units of agglutinin firmly enough to resist moderate washing with distilled water, or 12 to resist exposure to a pH within the range 7·0 to 3·0. Agglutinin in excess of this amount was easily dissociated from the agglutinated mass. Experiments with anti-typhoid sera and homologous antigen show that the constant-antigen optimum is in the region of a serum concentration 16 to 32 times that required to produce an end-titre. These facts suggest that the proportion of serum for full "saturation" of a given bacillary suspension is the optimal particulating proportion of the constant-antigen titration. The factor for *Brucella* agglutination is 16

and for *Bact. flexneri* agglutination about 30 (deduced from Costa Cruz' protocols). It is probable that it will vary with each type of suspension and serum.

The constant-antiserum titration has no theoretical end-point. Practically, a reading can be made with suspension as dilute as 300×10^6 per c.c. As the concentration increases from this limit, the speed of agglutination increases, since there is an increase in the probability of each sensitized bacterium coming in unit time to within a distance of a neighbour over which the " cohesive force " (Northrop and de Kruif, 1922) is active. A point is reached at which the amount of agglutinin per bacterium is insufficient to sensitize each bacterium fully, so that although the probability of mutual approach is greater, the cohesive force is less effective in holding aggregates of bacteria and the flocculation rate drops. The optimum represents a point at which flocculation rate is at a maximum, *i. e.* when the insufficient sensitization balances the effect of crowding of the bacteria.

Eventually the amount of agglutinin per bacterium is insufficient to sensitize at all, and complete inhibition results.

The Relation Between the Two Optima.

The agglutination velocity curves show that for a fixed dose of antigen, the ratio of the amount of serum in constant-antiserum optimum to that needed in a constant-antigen optimum is in the region of 1 : 4. More exact determinations verify this figure for the *abortus* and the *melitensis* sera with homologous antigens.

The ratio of the two optima in the precipitin reactions is of a lower order. Taylor (1933) finds 1 : 1·6 for crystalline egg albumen and its antiserum and 1 : 3 for a mixed antigen like horse-serum and anti-horse-serum. A study of the Ramon toxin-antitoxin curves in Fig. 4 shows that here the ratio is even smaller, and the constant antiserum optimum (the point at which a given constant antigen curve crosses to the left of the more concentrated-antigen curves) occurs at an antitoxin dilution very little greater than that required for the Ramon optimum in the curves for more dilute toxin, 1 : 1·3, while in those for stronger toxin the two optima appear coincident. Exact determinations of the ratio have yet to be made ; numerical deductions from the curves in Fig. 4 are probably unreliable.

The significance of the ratios between the two titration values depends on the exact determination of the equivalent tube. Hitherto excess of agglutinin in supernatant fluids has been titrated by agglutinating suspensions which are extremely insensitive to small concentrations of antibody. It may be that neither optima are equivalent, but represent equilibrium points between opposing factors that are only indirectly connected with specific antigen-antibody reactions at the bacterial surface, and in that case the hypotheses, presented above, of the production of optima break down. Assuming on the basis of the evidence here presented that the equivalent mixture in agglutination is the constant-antigen optimum, the ratio is a measure of the difference between, on the one hand, the minimum number of antigenic " groups " on each bacterial surface which must be satisfied before the suspension is fully sensitized to the

action of electrolytes, and, on the other hand, the total number of " groups " available for association with whatever specific agglutinins there are in the antiserum.

The Findings in the Brucella *Group.*

The following speculative explanation of the findings in the *Brucella* group is based on the assumption that both the interpretation of the optima given above and the antigenic picture of Wilson and Miles (1932) are correct. These authors pointed out that the serological behaviour of the group could be explained by assuming that each of the three types contained varying amounts of two antigenic components, " A " and " M ". *Br. melitensis* was for the most part M, with a little A ; the bovine *abortus* A, with a little M ; while the porcine *abortus* occupied an intermediate position, differing only a little from the bovine *abortus* in containing more of the M component. The optimal proportion titrations carried out fail to reveal any significant difference between the bovine and porcine *abortus*, and they may be considered as identical for the purposes of this discussion.

The constant-antigen titration.—The position of the optimal ratio depends on the proportions of the agglutinins to the different antigens on the bacterial surface. If the several components gave rise to agglutinins in the rabbit serum in such a way that their proportional distribution was the same as the proportional distribution of the components at the bacterial surface, a serum concentration containing optimal ratio of agglutinins for one component would contain the optimal ratio for all the other components. Thus one optimum only would be produced—a result of all the antigen-antibody reactions in the system.

In the course of immunization of a rabbit with a bacterium such as *Br. abortus* containing two antigens, one markedly in excess of the other, the response to the more abundant antigen will be absolutely greater. But late in the course of immunization further injection of this antigen will give rise to little or no increase in the amount of its agglutinin, while the stimulus of the less abundant antigen will still be effective. Consequently the serum contains a proportionately larger amount of agglutinin for the lesser component, though the absolute amount is less than that of the agglutinins for the main component. Titration of such a serum against a homologous suspension will yield two optima, for the more proportionately abundant agglutinin will be able to saturate its appropriate antigen in a lower serum dilution than that required for saturation of the other antigen.

The M agglutinin will be the more proportionately abundant in an *abortus* serum, the A agglutinin in a *melitensis* serum. That is, in a homologous constant-antigen titration the " lower " optimum is produced by the *heterologous* agglutinin.

Titration against a heterologous organism, in which the proportional distribution of the two components is different, disturbs the proportionality between the several agglutinins and their antibodies. If, for example, an *abortus* serum is titrated against a *melitensis* suspension, the A agglutinins will be in marked excess of the small A component, compared with the M agglutinins available for the large M component. As before, the lower optimum is produced

by the proportionately more abundant agglutinin, but in this titration it is the A agglutinin in *abortus* serum and the M in a *melitensis* serum, *i.e.* the *homologous* agglutinin.

The direct comparison of homologous and heterologous lower optima as shown in Tables II and IV is consequently invalid; different antigen-antibody systems are concerned.

There are many possibilities inherent in this explanation. If there are two optima present in homologous titration, the use of a heterologous suspension differing quantitatively in a small degree leads to their approach and final merging in a titration series; while a markedly different strain leads to the transposition of optima postulated above. It is thus purely fortuitous that the *abortus* and *melitensis* organisms are differentiable by the constant-antigen titration, for their antigenic distribution might have been such that an exact transposition of the two optima occurred in the change from homologous to heterologous titration, giving two apparently identical results.

The constant-antiserum titration.—The same reasoning holds for the reverse method of titration. Here the proportionately more abundant antigen produces the optimum in the region of most concentrated suspension. The optimum in the region of lower concentrations of suspension corresponds to the " upper " optimum of the constant-antigen titration. The non-appearance of two optima in the constant-antiserum titrations is probably due to the limited range of suspension concentrations ($10{\cdot}0 \times 10^9$ to $0{\cdot}3 \times 10^9$ per c.c.) available in the test. If one optimum is seen within this range, the other will be outside it.

SUMMARY.

1. The velocity of agglutination in a constant volume of fluid is at a maximum when the ratio of the concentrations of bacteria and immune serum has one of two values, depending on the method of titration. These values are constant, and bear a constant relationship to one another for a given antiserum and antigen.

2. One ratio is determined by titrating antigen against a constant antiserum concentration (the " Dean and Webb " method), the other by titrating antiserum against a constant antigen concentration (the " Ramon " method). The significance of and the relations between the two ratios are discussed.

3. Secondary optimal ratios of as yet unknown significance in the constant-antigen titrations are described.

4. Representative smooth strains of *Br. abortus*, bovine variety, and *Br. abortus*, porcine variety, are distinguishable from *Br. melitensis* by use of the optimal proportions technique. The *abortus* varieties are indistinguishable from one another. The limitations of the method applied to antigenic analysis of bacteria are discussed.

It is a pleasure to record my thanks to Prof. G. S. Wilson for supplying the strains of *Brucella* used; to Dr. G. L. Taylor for a specimen of horse-serum albumen; to Dr. G. F. Petrie for the Ramon test reagents; and to Dr. Wilson Smith for the technique of squirt-mixing in the precipitin tests.

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